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BIOCHEMICAL ASPECTS OF MICROBIAL PATHOGENICITY

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BIOCHEMICAL ASPECTS OF MICROBIAL PATHOGENICITY*

Conference Editor: WERNER BRAUN

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INTRODUCTORY REMARKS

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In the spring of 1960, it is hardly necessary to justify the statement that some of the discoveries of modern biology have come, and many more undoubtedly will come, as a result of an emphasis on what has become known as molecular biology. The present monograph represents the result of many years of personal interest in assembling in one publication our current knowledge of molecular mechanisms as it pertains to problems of microbial pathogenesis.

Tropical diseases have always been of great interest to me, but my interest was brought into focus during my stay at Johns Hopkins School of Hygiene and Public Health, Baltimore, Md., in 1956. Since then I have wanted to see something like the present monograph published. This monograph has, however, materialized more as a result of Werner Braun's energy and realistic approach than as a result of my imagination.

I earnestly hope that this publication will serve not only to integrate our existing knowledge of the molecular mechanisms of microbial pathogenesis but also will serve to stimulate wider interest in this field, particularly among biochemists.

Part I. Biochemical Properties of Virulent and Avirulent Strains of Bacteria

BIOCHEMICAL PROPERTIES OF VIRULENT AND AVIRULENT STRAINS OF *BACILLUS ANTHRACIS*

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Although anthrax was the first disease shown to be caused by bacteria, there is much that we still do not understand about the disease and the responsible organism *Bacillus anthracis*. In many areas a great deal of information has been accumulated about the organism, such as nutritional requirements for growth, factors affecting capsule formation, some conditions required for toxin production, and factors affecting sporulation. However, in spite of all this knowledge there are many unsolved problems concerning its virulence. To attest to this fact it is necessary only to point out that the only way to grade cultures with respect to virulence is to test them in animals. Within certain limits strains can be screened by presumptive tests *in vitro*, but confirmation can be attained only by animal tests.

Two factors that almost certainly are associated with virulence are the capsule and toxin. This report consists mostly of summaries of the current status of our knowledge about each of these factors.

Glutamyl Polypeptide and Capsule Formation

All virulent strains as well as some avirulent strains of *B. anthracis* produce a capsule composed largely, if not entirely, of a polypeptide of glutamic acid. For some time our Microbial Physiology group at Fort Detrick has been interested in factors affecting capsule formation and glutamyl polypeptide synthesis by *B. anthracis* and *B. subtilis*. With *B. anthracis* the peptide occurs in the capsule and is released upon autolysis or by autoclaving the cells, whereas with *B. subtilis* the peptide occurs free in the medium.

This subject was reviewed recently[1] and much of the detailed information will not be repeated here.

There is no doubt that glutamic acid is the only amino acid released upon acid hydrolysis of the peptide, but there are conflicting reports as to the configuration of the glutamic acid. Ivanovics and Bruckner[2] showed that D-glutamic acid was produced upon acid hydrolysis of polypeptide from *B. anthracis* and *B. mesentericus*. Bovarnick[3] concluded that polypeptide from *B. subtilis* was composed solely of D-glutamic acid, but Watson *et al.*[4] found that only 85 per cent of the glutamic acid from polypeptide produced by *B. subtilis* was the D-isomer. The peptide preparations from *B. anthracis* studied by Hanby and Rydon[5] contained only D-glutamic acid.

My associates and I[6] have shown that synthesis of glutamyl polypeptide by *B. subtilis* during growth in a synthetic medium is dependent on the metallic ion composition of the medium. Without any change in the total yield of peptide, the proportion of D-glutamic acid in peptide of culture filtrates varied from 38 to 86 per cent with increasing concentrations of Mn^{++}, Co^{++}, or Zn^{++}. Further experiments[7] showed that *B. subtilis* produced two glutamyl polypeptides, one composed predominantly, if not entirely, of the D-isomer and the other similarly composed of the L-isomer. The isolated D- and L-peptides possessed the peculiar characteristic of coprecipitating from acid solution, although separately they were each soluble. This explained earlier observations that preparations of peptide from *B. anthracis* were soluble in acid while various preparations from *B. subtilis* were insoluble in acid. In contrast to our results with *B. subtilis*, our determinations have not shown any significant amount of the L-isomer in glutamyl polypeptide preparations from *B. anthracis*.

According to Torii,[8] glutamyl polypeptide from *B. megatherium* contains both D- and L-isomers and, based on its solubility in acid and on its immunological characteristics, Utsumi *et al.*[9] have concluded that it is a copolymer of D- and L-glutamic acids. However, such a conclusion based on immunological data needs to be confirmed because, by their acid nature, glutamyl polypeptide preparations can give nonspecific precipitates with basic substances in serum (unpublished observations).[10]

The best chemical evidence,[11,12,13,14] as well as information from experiments on specific enzymatic hydrolysis,[1] indicates that the glutamyl residues in the polypeptide are connected largely, if not entirely, through the gamma carboxyl groups.

Toxin

After a detailed study of anthrax in laboratory animals, Cromartie *et al.*[15,16] concluded that the histopathological changes observed could be best explained by the production of a diffusible poison, and they showed that crude extracts of anthrax lesions produced characteristic edematous reactions when injected intracutaneously into rabbits. The presence of a specific lethal toxin, which was related immunologically to the protective antigen and which was presumably the same as the toxic material originally found in anthrax lesions, was demonstrated by Smith *et al.*[17] in the plasma removed from guinea pigs immediately after death from anthrax. The toxin consisted of two components that could be partially separated by ultracentrifugation.[18] Initial attempts to produce toxin *in vitro* were unsuccessful, but eventually toxin was produced in a medium containing a large amount of serum.[19]

In our laboratory we[20] found that culture filtrates were toxic, as evidenced by a skin test in guinea pigs and a lethality test in mice, when *B. anthracis* was grown under conditions for protective antigen production,[21] either in a casamino acids medium or in a completely synthetic medium, or when the media were supplemented with either 10 per cent v/v of normal serum or 1 per cent w/v of gelatin. However, culture filtrates obtained after growth of the organism in either medium without added protein were completely nontoxic, although the protective antigen titers were equivalent to those of toxic culture filtrates from media containing serum or gelatin. It seemed probable that if the toxin consisted of two components, as shown by Smith *et al.*,[18] and one of them was the protective antigen, then in the absence of serum or gelatin the other component was either (1) not produced, (2) destroyed as soon as it was formed, or

(3) removed or inactivated during filtration of the growth medium. Possibilities 1 and 2 were eliminated by the finding that the addition of serum to culture fluid after growth and just before filtration resulted in a toxic filtrate. Further experiments showed that a component of toxin was adsorbed on sintered glass filters and could be eluted with alkaline buffer. For convenience this component is referred to in our laboratory as filter factor. TABLE 1 gives an example of the method used for preparing filter factor.

Preparations of filter factor, as well as preparations of protective antigen, were completely nontoxic when tested alone, but when the two were combined they formed a toxic mixture. On agar diffusion plates with antiserum prepared in a horse against the Sterne (Weybridge) strain, filter factor preparations obtained by elution from glass filters produced two lines of precipitation that were different from the line of precipitation given by protective antigen

TABLE 1

PREPARATION OF FILTER FACTOR COMPONENT OF *B. ANTHRACIS* TOXIN*

Fraction	Volume ml.	Toxicity† (guinea pig skin test)	
		Units per ml.	Total
1*a* Culture supernatant fluid	2200	128	281,600
1*b* Filtrate from 1*a*	2200	0	0
1*c* Eluate from glass filter used to obtain 1*b*	20	12,800	256,000

* The Sterne (Weybridge) strain was grown in 3-l Fernbach flasks with 500 ml. of Casamino acids medium.[20]

† To determine toxin units in culture supernatant fluid, 10 per cent v/v of horse serum was added to a sample that was then sterilized by filtration through sintered glass and assayed. The addition of serum prevented the adsorption of filter factor. The eluate (filter factor) was completely nontoxic when tested alone but formed toxic mixtures with protective antigen. For assay purposes the filter factor was diluted in gelatin-phosphate diluent containing 4 μg. protective antigen per ml.

Attempts to separate the antigens responsible for the two lines by ammonium sulfate fractionation were unsuccessful. However, recent experiments on fractionation with ethanol have been more fruitful. Preparations of each constituent free from the other have now been obtained. One of the constituents has no toxic activity either alone or when mixed with protective antigen. Antiserum prepared against it had no effect on toxicity of active preparations of filter factor. The other constituent appears to be the one responsible for toxic activity. It too is nontoxic when tested alone, but it forms toxic mixtures with protective antigen. There is good correlation between the titer of this material as determined by the agar diffusion method of assay[21] and the toxin titer of preparations. As yet antiserum to this isolated component has not been prepared. There is evidence that the inactive constituent is a degradation product of this active substance.

Rabbits immunized with preparations of filter factor were protected against the disease. Protection tests have not yet been done with the isolated component of filter factor that correlates with toxicity. However, rabbits immunized with the other inactive component were not protected against the disease, and therefore the component responsible for the toxicity appears to be also responsible for the immunity. Thus there are at least two protective antigens produced by *B. anthracis*. Since the toxin is made up of two antigenic components, the presence in an animal of antibody to either should result in neutralization of toxin and protection against the disease.

Our results are in agreement with the findings of Smith *et al.*[18] with respect to the presence of two components in the toxin of *B. anthracis*. In their description of conditions for the production of toxin *in vitro*, Harris-Smith *et al.*[19] report that a trace of toxin was produced in tryptic meat-broth medium but that higher and more reproducible yields were obtained when 90 per cent v/v of serum was included in the medium. They suggested that the large molecular components of serum play an important part in toxin production. Our studies show unequivocally that serum is not required for toxin production and failure to recognize this earlier is due to the fact that filtration of growth medium to free it from bacteria results in the removal of one of the toxin components. When serum or other protein is present, presumably the factor is preferentially adsorbed by it and thus passes through the filter or perhaps the added protein saturates or otherwise blocks the adsorption capacity of the glass, allowing the toxin component to pass through.

There is another manner in which serum can function to allow accumulation of toxin in a growing culture. In our studies leading to purification of protective antigen from culture filtrates of *B. anthracis*, we learned that culture filtrates contained an enzyme or enzymes that degraded the antigen.[22] The enzymatic degradation was inhibited by maintaining a high *p*H or by addition of serum or gelatin. Similar results have also been found with filter factor preparations. At *p*H 7 or lower, culture filtrates rapidly degrade isolated filter factor. The degradation can be followed by determining the loss in toxic activity as well as by visual examination of the lines of precipitation on agar diffusion plates. The enzymatic activity is reduced at a high *p*H (*p*H 8 to 9) or in the presence of serum or gelatin. Thus serum can contribute to the accumulation of toxin in a culture by preventing its destruction. However, if enough bicarbonate is added to the medium to result in a sufficiently high *p*H, the enzymatic degradation of both components of the toxin is inhibited and the addition of serum is not necessary.

Serum is often added to culture media to simulate *in vivo* conditions. The finding that serum is not essential for toxin production provides another example to support our assumption that neither the use of serum *in vitro* nor actual cultivation *in vivo* is essential for any particular metabolic activity of an organism.

Most of our studies on toxin have been done with the avirulent Sterne (Weybridge) strain. However several other strains, virulent as well as avirulent, have been tested and found to produce toxin. Although there is no obvious correlation between the relative virulence of strains and their capacities to produce toxin, a thorough quantitative study has not been made. We have found that conditions that are optimum for toxin production by one strain are not necessarily optimum for other strains. Therefore, before toxin-producing capacity could be correlated with virulence, optimum conditions for all strains under study would have to be worked out.

B. cereus also produces toxin when grown under the same conditions used for production of toxin by *B. anthracis* (unpublished observations). Four strains have been tested in our laboratory and all gave similar results, but filtrates from cultures of *B. subtilis* and *B. megatherium* were inactive. Although some of the conditions required for toxin production by *B. cereus* are similar to those required by *B. anthracis*, the toxins of the two organisms appear to be very different. Toxic filtrates from each organism give skin reactions in guinea pigs and both are lethal to mice. However, the skin reaction given by *B. cereus* toxin is strongly necrotic as well as edematous; little or no necrosis is caused by the toxin of *B. anthracis*. Also, *B. cereus* toxin kills mice much faster (10 to 15 min.) than anthrax toxin (18 to 120 hours). The reactions given by *B. cereus* toxin are neutralized by homologous antisera but not by antiserum to *B. anthracis* or to toxin of *B. anthracis*. The only similarity we have found is that, like the anthrax toxin, the *B. cereus* toxin also appears to have two components. Unlike the filter factor of the anthrax toxin, neither of these components is adsorbed on glass filters, but the two can be separated by fractionation on columns of calcium phosphate.

Chu[23] reported that preparations of lecithinase from *B. cereus* were dermonecrotizing when injected intradermally into rabbits and guinea pigs and lethal when injected intravenously into mice. We believe that the toxic principle of *B. cereus* that is under investigation in our laboratory is not lecithinase since there appears to be no correlation between lecithinase activity and toxicity of various fractions.

For those interested in the taxonomic classification of *B. cereus* and *B. anthracis*, toxin production should be a useful property to add to the list of distinguishing characteristics. The two toxins could hardly be mistaken one for the other. Burdon and Wende[24] showed that the disease caused by injection of actively multiplying strains of *B. cereus* into mice, guinea pigs, or rabbits is very different from anthrax. Their results are in accord with the fact that the two species produce different toxins.

The mechanism of action of anthrax toxin has yet to be ascertained. According to Smith[25] the toxin acts as an aggressin in the early stages of the disease since it inhibits phagocytosis of *B. anthracis* and also inhibits the action of an anthracidal factor present in some sera and in extracts of leukocytes. Smith *et al.*[17] reported that the cause of death in guinea pigs dying after injection of toxic plasma obtained from infected guinea pigs was secondary shock with renal failure and that the syndrome was identical to that found in animals dying of infection with *B. anthracis*. There was an increase in the alkaline phosphatase of blood plasma while kidney alkaline phosphatase was decreased. Hyperglycemia also occurred.

Slein and Logan[26] in our laboratory have undertaken to determine the biochemical lesion or lesions produced by the toxin. Increases in serum aldolase, alkaline phosphatase, phosphoglucose isomerase, and glutamic acid-oxaloacetic acid transaminase occurred in mice within 24 hours after a lethal dose of toxin was injected intravenously. Transient hyperglycemia was detected less than one hour after the injection of toxin. Similar changes in serum constituents were found with rabbits. Glucose and alkaline phosphatase increased rapidly and markedly. Production of hyperglycemia required both components of the toxin, but hyperphosphatasemia was produced by the injection of protective antigen alone as well as in combination with filter factor. It did not occur when only filter factor was injected. Bilateral nephrectomy did not prevent the hyperphosphatasemia produced by injection of the protective antigen component of the toxin. Thus in rabbits the increase in serum alkaline phosphatase does not result entirely, if at all, from kidney damage.

Variation in B. anthracis

Sterne[27] observed that, with respect to virulence and capsule formation, most strains of *B. anthracis* fall into 1 of 3 types. These observations were extended by Chu[28] and by Thorne *et al.*[29] and are summarized in TABLE 2. Virulent strains (S type) produce capsules of glutamyl polypeptide when grown *in vivo* or when grown *in vitro* in the presence of bicarbonate and in an atmosphere rich in CO_2. Colonies of capsulated organisms are mucoid and can be easily distinguished from the rough colonies of noncapsulated cells. A second type, which is avirulent and occurs rarely, produces capsules and mucoid colonies when grown in the absence of added bicarbonate or CO_2 and their addition has little or no effect. A third type (R type), which is also avirulent, does not produce capsules under any conditions and thus always gives rough colonies.

TABLE 2
TYPES OF *B. ANTHRACIS*

Type	Forms capsules in		Colonial morphology in		Virulence
	CO_2	Air	CO_2	Air	
I (*S*)	+	−	mucoid	rough	+*
II	+	+	mucoid	mucoid	−
III (*R*)	−	−	rough	rough	−

* This is the normal virulent type of *B. anthracis*. However, certain "attenuated" strains occur that fall into this type with respect to capsule formation but are of relatively low virulence. Presumably they have reduced capacity to produce toxin.

The R type arises spontaneously from the S type and all virulent cultures that have been examined contain at least a small proportion of the R type. The S → R variation can be observed among colonies of the S type on nutrient agar containing bicarbonate and incubated in an atmosphere of 10 to 75 per cent CO_2 in air. Colonies of the S type appear mucoid but, upon prolonged incubation, occasional colonies will produce a rough outgrowth. When such rough outgrowth is subcultured either in air or in CO_2, it produces colonies of the avirulent R type. Culturing the organisms in a bicarbonate medium under CO_2 offers a selective advantage for the R type, and broth cultures originating from the S type but grown under these conditions may contain from <1 to >90 per cent of the R type depending on the age of the culture and on the strain. This phenomenon occurs more readily with some strains than with others.

The change from the virulent S type to the avirulent R type has been known for several years to occur *in vitro* and it has recently been shown in our laboratory that this change can also occur *in vivo*.[30] A large proportion of the organisms in tissues and fluids of animals dead from anthrax may be the R type. The proportion of the R type isolated from dead animals varies with the strain and increases with the survival time of the animal. The longer the animal survives after infection, the greater the proportion of R-type cells in the tissues.

Since R-type cells are able to produce toxin, once they become established in the host they should contribute to the progress of the disease in the same manner as S-type cells do. Their inability to produce capsules apparently renders the R type avirulent because it cannot become established in a healthy host. However, after the S type has caused damage to the host's tissues, then, as R-type cells occur, they are apparently able to survive and produce toxin.

TABLE 3

ISOLATION OF S-TYPE CELLS FROM A MIXED POPULATION OF S AND R TYPES*

No. of cells added per plate		Phage added	Incubation in air or CO_2	Number and type of colonies
S type	R type			
25	0	no	air	20 rough
25	0	yes	air	0
25	0	yes	CO_2	19 mucoid
0	2×10^7	no	air	confluent rough growth
0	2×10^7	yes	air	many isolated rough colonies
0	2×10^7	yes	CO_2	many isolated rough colonies
25	2×10^7	no	CO_2	confluent rough growth
25	2×10^7	yes	CO_2	11 mucoid, many isolated rough colonies

* Petri plates contained 25 ml. of nutrient agar with or without 0.5 per cent $NaHCO_3$, and 0.1 ml. of S, R, or mixed spores with or without 0.1 ml. of phage was spread over the agar surface. Plates were incubated 24 hours at 37° C. in air or 30 per cent CO_2 as indicated.

Schaeffer[31] reported that occasionally rough variants would again produce capsules, and he postulated that there were two kinds of rough variants, a stable type and an unstable type. Sterne[32] suggested that Schaeffer might not have succeeded in isolating rough variant cultures that were homogeneous, that is they might have been contaminated with the S type. At any rate Sterne did not observe a reversion of R to S, and I am not aware of any other report of such reversion although various investigators have looked for it. However, it is unlikely that, with the usual techniques of growing the organism, a reversion could be detected if it did occur. There is no difficulty in isolating avirulent, noncapsulated R-type cells when they are mixed with a population of virulent, capsulated S-type. The technique for such isolation, as implied above, consists of plating the culture on agar medium containing 0.5 per cent $NaHCO_3$ and incubating in an atmosphere of approximately 30 per cent CO_2 in air. Under such conditions the S-type cells form mucoid colonies and the R-type form rough colonies. It is not difficult for even inexperienced workers to distinguish between the two types. The rough cells have a selective advantage under such conditions and thus form visible colonies even when they represent a small proportion of the total population. However, since under these conditions of growth the R type does have selective advantage over the S type, the R type overgrows the S type when the latter represents a small proportion of the total population and mucoid colonies do not become apparent.

McCloy[33] made the observation that noncapsulated cells of *B. anthracis* are lysed by phage while capsulated cells are resistant. We have developed a method, which is based on this observation, for detecting S-type cells. As few as 1 to 10 S cells in the presence of 10^8 or more R cells can be detected by mixing the organisms with phage and culturing on bicarbonate agar in CO_2. The S cells become capsulated and are not lysed by the phage. The R cells are lysed, allowing the S cells to form visible mucoid colonies. Some colonies of R cells that escape lysis by the phage usually appear on plates prepared in this manner, but these represent a small proportion of the total population and do not interfere with development of mucoid colonies by S cells. TABLE 3 gives examples of results obtained with this procedure when prepared mixtures of S and R types were used. The phage used in these experiments was one isolated in our laboratory from soil.

Results of preliminary experiments with the phage technique indicate that R to S reversion does occur. Confirmatory experiments are now in progress.

General Discussion

In one of his publications Sterne,[27] in discussing the S to R variation in *B. anthracis*, proposed that the virulence of the organism is determined by the capsule and by another factor not associated with capsule that he, for convenience, termed "A factor." He further proposed that: (1) the immunity to anthrax produced by any particular strain is due to "A factor"; (2) a strain may become avirulent by losing the ability to produce "A factor," although retaining the ability to form capsules; and (3) a strain may become avirulent by losing the ability to form capsules although retaining the ability to produce "A factor." These observations and theories were proposed in 1937 to explain virulence of *B. anthracis* and properties of variant types. No new concepts or findings since that time have been reported to further our understanding of anthrax. To be sure many workers have obtained pieces of information that support the proposals of Sterne. Thus a proposal to explain virulence of *B. anthracis* based on current knowledge is really no different from that of Sterne, although certain aspects are now supported by more substantial evidence. Undoubtedly the two primary factors concerned with virulence are the capsule and toxin, and protection from the disease by immunization is based on the antigenicity of the toxin components. To be virulent a strain must have the ability to produce capsules as well as toxin; a loss of either property results in loss of virulence.

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Discussion of the Paper

H. Smith (*Microbiological Research Establishment, Porton, England*): A rise in serum alkaline phosphatase is produced by injection of a number of bacterial toxins, including endotoxin and the α-toxin of *Clostridium welchii*. A similar rise in this enzyme occurs in ischemic shock. Hence the increase of this enzyme appears to be one of the general reactions of the body to injury. There is a possibility of a more specific action in anthrax since the rise in serum phosphatase is large; much of the enzyme comes from the kidney since there is a gross loss from this tissue.

What precisely is your evidence that the increased serum alkaline phosphatase is specific to the anthrax toxin and not one of the general reactions of the body to injury?

Thorne: There is no conclusive evidence that the increase in serum alkaline phosphatase is a primary and specific effect of the toxin of *B. anthracis* and I did not intend to imply such a conclusion. The cause and specificity of this effect and the source of the enzyme are still under investigation. Your reference to loss of alkaline phosphatase from the kidney is presumably from experiments with guinea pigs. Perhaps one should be cautious in assuming that the enzyme disappearing from the kidney is the same enzyme, that is, the same molecules of enzyme protein, that appears in the serum. There is also the possibility that the mechanism of response to toxin in the rabbit is different from that in the guinea pig. Bilateral nephrectomy did not prevent the hyperphosphatasemia in rabbits, but this was not tested in guinea pigs.

BIOCHEMICAL PROPERTIES OF A VIRULENT AND AN AVIRULENT STRAIN OF GROUP A HEMOLYTIC STREPTOCOCCUS

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In this monograph each contributor discusses the problem of the identification of biochemical parameters that underlie the virulence of the particular microbe that he has studied. How readily the experiences of individual investigators working with different microbes can be translated into some general concepts for understanding the kinds of biochemical variation that influence virulence will depend on a general acceptance of what is meant by virulence. I propose to begin by defining my subject in reference to the virulence of the group A hemolytic streptococci. To begin with, the data that I shall discuss will come primarily from experiments with mice. Consequently I shall be discussing the virulence of group A streptococci for mice, and even this subject represents a generalization, since my primary experience will be relative to a type-14 group-A streptococcus and its parent culture, which is untypable. The ability of these strains to kill mice when given by the intraperitoneal route as a function of the infecting dose is the biological phenomenon that I am equating with virulence in this discussion, and an attempt will be made to assess the biochemical and other differences that have been observed in these two strains in relation to their virulence.

Naturally, there is only very limited value in studying the death of mice given inoculations of streptococci by a highly unnatural route. Such studies can be justified as the need to satisfy a vagrant curiosity concerning a specific biological phenomenon. However, I presume that most of us are interested in what our studies will tell us concerning the natural ways in which microorganisms cause infections in animal hosts and especially in man. If we are to understand the properties of the group A streptococci that allow them to parasitize man successfully, bringing about acute pharyngitis, nephritis, erysipelas, scarlet fever, or the rheumatic processes, then the principal instruction must come from observations of people who are ill with or recovering from contact with the streptococcus. However, a proper dissection of the variables of a biological phenomenon requires experimental conditions that allow for controlled observation and manipulation. Thus we all turn to mice, monkeys, guinea pigs, or other animals. Nevertheless, we must be aware that in going from man to animals and in imposing artificial routes of giving quantities of organisms grown under defined, but nevertheless artificial conditions, we have greatly changed the context of the biological phenomenon we are investigating. We can still talk about the virulence or pathogenicity of an agent, but of course it may now have an altered meaning. However, it is a general conviction that useful information can be obtained under these circumstances which, if interpreted skillfully in relation to the findings from clinical situations and knowledge about the natural history of specific infectious processes, can lead to a significant extension of the sophistication with which we can think about disease processes in man.

Characteristics of Q Cultures

My associates and I have chosen an experimental situation based on the early observation of Todd (1928) and of Todd and Lancefield (1928) that variants of streptococci could be found that were avirulent for mice and that were distinguishable from virulent cultures by a glossy colonial morphology, whereas the virulent strains formed colonies described as matt. A further correlation was noted by them, namely that the glossy, avirulent colonies lacked a characteristic M protein, whereas the matt, virulent organisms contained a serologically identifiable M protein. These considerations can best be reviewed by looking at the characteristics of the cultures that we employed in our studies (Leedom and Barkulis, 1959). Through the kindness of Eugene Fox of Western Reserve University, Cleveland, Ohio, a culture designated as Q496 was made available to us. This culture had been isolated by throat swab from an individual convalescing from streptococcal pharyngitis. As is frequently the case with group A streptococcal isolants from such clinical situations, this culture was relatively avirulent for mice. We mouse-passed the Q496 culture. Most of the data I shall discuss will be a comparison of the Q496 culture with a strain derived from it after 43 mouse passages, and which will be designated as Q43x. Some of the characteristics of these two strains are compared in TABLE 1. Conforming to Todd's and Lancefield's observations, the Q496 culture forms glossy colonies on blood agar that are surrounded by a zone of β-hemolysis. Young colonies are mucoid. The LD_{50} dose in mice is 500,000 chains. It has group A serologic characteristics but cannot be typed with the known type-specific antisera. By contrast, the Q43x culture has a matt colonial morphology. This, however, is not as mattlike as other mouse virulent cultures we have in the laboratory. Young Q43x colonies are also mucoid in appearance indicating the presence of capsular material. It differs from the Q496 culture in that it is markedly mouse-virulent with an LD_{50} dose of 25 or fewer chains. In addition to serologic characteristics of group A, it is type-14 specific.

TABLE 1
CHARACTERISTICS OF Q CULTURES

Culture	Colonial morphology	Plate hemolysis	Capsule	Mouse passages	LD_{50} (mice)	Serologic	
						Group	Type
Q496	Glossy	β	+	0	500,000	A	—
Q43x	Matt	β	+	43	25	A	14

A word might be said here about the colonial morphology of group A streptococci. Three colony types are recognized and have been designated mucoid, matt, and glossy. Wilson (1959) has helped to clarify the basis of colonial differences in a recent study. Our observations on the Q cultures differ with his conclusions in only one respect. If colonies of the Q496 and Q43x cultures on blood agar are observed within 18 hours after seeding the plate, both appear mucoid. The Q496 glossy strain differs from cultures described as glossy by

Wilson in that his did not produce capsules and were apparently never preceded by a mucoid colonial phase. An apt description of a mucoid colony has been "dewdrop" in appearance. These are glistening and very smooth in surface. This mucoid appearance is due to the presence of capsules. However, on subsequent reincubation of the plates, the capsular material is apparently hydrolyzed. In any event it disappears, and the colonies take on a glossy or matt appearance. Swift (1952) has described the glossy colonies as having a "dry, glistening surface, like shiny photographs." This is true of the Q496 colonies. Swift also described the matt colony as having a "granular, nonglistening surface which resembles that of a nonglossy photographic print." The Q43x colonies can be so characterized after the capsules have disappeared. However, as stated above, the mattlike appearance of the Q43x colonies is not nearly as striking as that of other virulent strains that we maintain in the laboratory, such as S23. It should also be emphasized that the relationship of avirulence, glossiness, and lack of M protein distinguished from virulence, mattiness, and M protein content is generally a valid one. There are, nevertheless, known cultures that cannot be readily distinguished in these terms. Relatively avirulent, M-containing, mattlike cultures are known, and I have little doubt that virulent M-containing cultures whose colonies are more glossy than mattlike can also be found.

TABLE 2

METABOLIC CHARACTERISTICS OF Q CULTURES

Culture	Q lactic acid*	Growth rate (*in vitro*)	Streptolysin S units	Streptolysin O units/ml. of culture supernatant	DPNase	Arginine dihydrolase
Q496	322	+++	1500	2.3†	0	+++ (Fox)‡
Q43x	220	+++	200	1.0†	0	+++ (Fox)‡

* Microliter lactic acid produced from glucose per hour per mg. dry weight of organism.

† Determined by Leedom for Q 496 and a 22× mouse-passed type-14 culture derived from it.

‡ Determined by Fox for Q 496 and a mouse-passed (17×) type-14 culture derived from it.

Summarized in TABLE 2 are a number of metabolic characteristics of the Q cultures. On the whole, it may be said that the Q496 culture has a greater metabolic potential than that of the Q43x. Thus Q496 glycolyzes at a more rapid rate. Perhaps more surprising, under resting conditions it produces considerably more streptolysin S and also more streptolysin O; the DPNase production of both cultures is negligible. Fox (1955) has also looked at the arginine dihydrolase activity of the Q496 culture, and a type-14 culture derived from it after 17 mouse passages. He did not find any differences between the two strains in the amount of this enzyme. It was a somewhat surprising finding to us that the capacity of the Q496 culture to produce hemolysin *in vitro* was greater than that of the Q43x culture. It might have been thought that the greater degree of virulence of the latter would have been associated with a greater capacity for the production of these toxins. Nevertheless, these *in vitro* results do little more than indicate the capacity for lysin production under optimal circumstances in a resting state. FIGURE 1 shows two plates, seeded respectively with Q496 and Q43x organisms. After 24 hours we regularly find that the zone of hemolysis around the Q43x colonies is substantially greater than that around the Q496 colonies. This finding is not consistent with the capabilities of these cultures for hemolysin production in a resting state. The lesson would seem to be that it is dangerous to attempt to relate virulence of cultures to their capability for toxin production under defined *in vitro* situations.

Susceptibility of Q Cultures to In Vitro Phagocytosis

We now may consider what appears to be an important property in the relative abilities of these two cultures to establish infection, that is, their susceptibility to phagocytosis. The literature on the relationship of phagocytosis to virulence of the group A streptococci extends over 60 years. The conclusions of early workers (Bordet, 1897; Marchand, 1898; Wallgren, 1899) from *in vivo* studies on peritoneal exudates from guinea pigs with experimental streptococcal peritonitis was that avirulent or "attenuated streptococci are readily destroyed by leukocytes, whereas virulent streptococci tend to resist phagocytosis" (Foley *et al.*, 1960). As the newer knowledge concerning the antigenic composition of the streptococci developed, it was tempting to ascribe the differing susceptibility to phagocytosis on the basis of the differences in M protein composition of the virulent with the avirulent strains (Lancefield, 1940–41). Succeeding studies (Rothbard, 1948; Morris and Seastone, 1955) have confirmed this relationship. This is readily demonstrated with our Q cultures in TABLE 3, which summarizes the findings on their susceptibility to phagocytosis when mixed with heparinized human whole blood and viewed under a phase microscope, in the presence of normal rabbit serum and of type-14 rabbit antiserum. The Q496 organisms are rapidly and readily phagocytized. By contrast, very few of the Q43x organisms are seen to be phagocytized. The leukocytes seem to ignore them. Armine Wilson has documented such observations by cinemicrophotography in a film that provides an exciting spectator experience. In the presence of type-14 antiserum, however, the Q43x organisms are also rapidly phagocytized. Neither culture is leukotoxic. The phenomenon of leukotoxicity has been restudied recently by Wilson (1957). It may be described as the rapid disintegration that is seen with human and some animal neutrophils shortly after they have ingested streptococci. This capacity is uniformly present in all strains of some types, such as 6 and 12, in none of the strains of other types, including 5, 14, and 30, and a few types have both leukotoxic and nonleukotoxic representatives. In a later publication, Bernheimer *et al.* (1957) showed an interesting association of the ability of strains to elaborate the enzyme DPNase that cleaves DPN and the property of leukotoxicity. The Q cultures fit this pattern in that they are not leukotoxic and produce negligible amounts of DPNase.

A further illustration of the susceptibility of the Q strains to phagocytosis can be seen in the results of the indirect bacteriostatic test (Rothbard, 1945). Briefly summarized, in this test we incubate 10^{-4}, 10^{-5}, and 10^{-6} dilutions of an 18-hour culture with heparinized human whole blood in stoppered tubes that are tumbled for 3 hours at 37° C. Standard loopsful are then streaked on plates and the number of organisms that grow out are quantitated in a rough

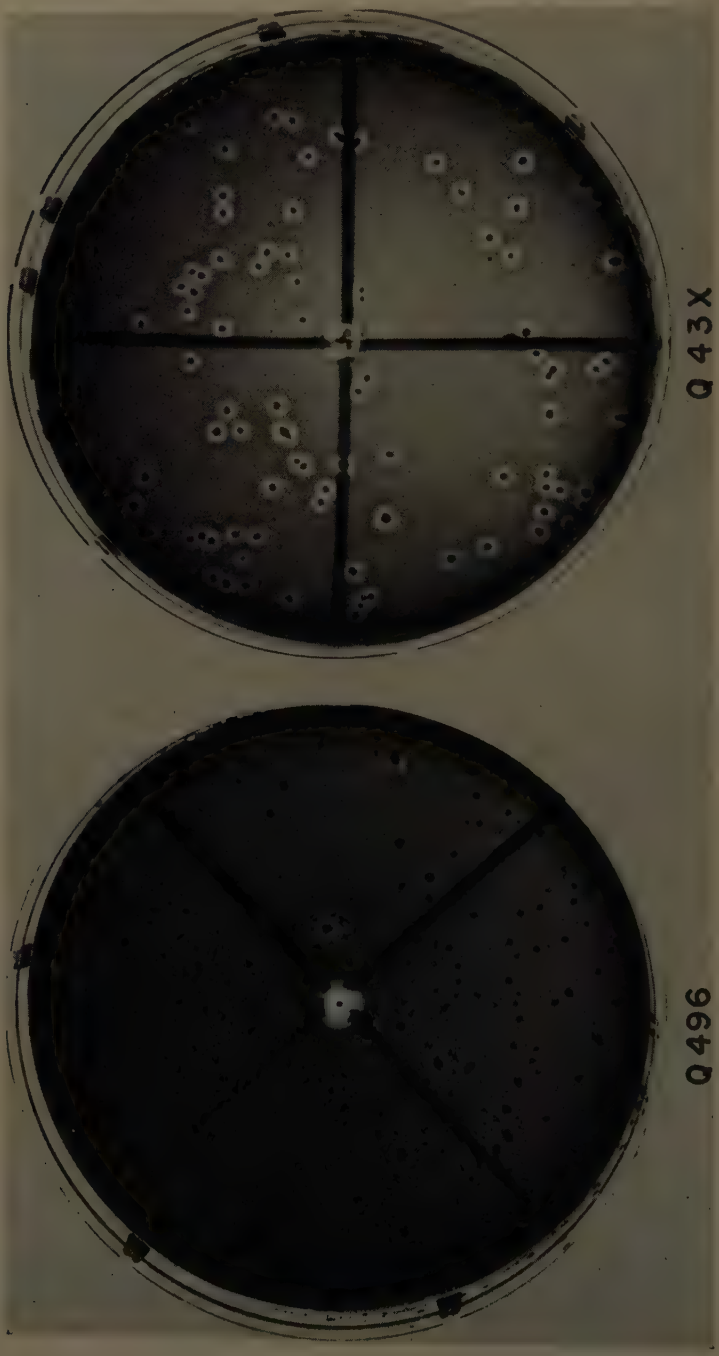


FIGURE 1.

manner. FIGURE 2 shows plates from such an experiment. The one on the left shows four +, three + and one + colonies respectively in the 10^{-4}, 10^{-5}, and 10^{-6} tubes. The plate to the right contained rabbit type-14 antiserum in addition, and it can be seen that only 6 colonies grew out from the 10^{-4} tube. Findings with the Q cultures are shown in TABLE 4. In one respect these are somewhat surprising since they show that the Q496 organisms, incubated with heparinized human whole blood in the presence of normal rabbit serum, apparently retain their viability during the course of the 3-hour incubation involved in the test, despite the fact that we have demonstrated under the microscope a ready phagocytosis of these chains. We do not understand the reasons for this survival. Wilson *et al.* (1957) have shown that streptococcal chains that have been phagocytized and been retained in the phagocyte for approximately 15 min. are no longer viable. However, he also described (Wilson, 1953) the phenomenon of egestion of chains and cocci from leukocytes that have previously phagocytized them. It may be that under the conditions of the test described here, the process of egestion is sufficiently frequent to allow the culture to remain largely viable in spite of extensive phagocytosis during the period of the test. In the presence of type-14 antiserum, it can be seen that there is some decrease in the viability of the avirulent culture. This is not striking, however. By contrast, the Q43x culture, which we know is poorly phagocytized in the presence of normal rabbit serum, multiplies during the course of the test and grows out in great numbers when streaked on plates. In the presence of type-specific antiserum, however, a rapid phagocytosis takes place which results in the non-viability of the culture with only few survivors in the lowest dilution of culture used.

TABLE 3

BEHAVIOR OF Q CULTURES WITH HUMAN LEUCOCYTES *IN VITRO*

	Phagocytosis in presence of		
Culture	Normal rabbit serum	Type-14 rabbit serum	Leukotoxicity
Q496	++++	++++	0
Q43x	0	++++	0

The role of the hyaluronic acid capsule of the group A streptococci in relation to phagocytosis has been the subject of varied opinion in the literature (Lancefield, 1954). Although most workers have agreed that the capsule has some antiphagocytic effect, the extent of this has varied in the experiments of different investigators. Wood and his co-workers have recently re-examined the problem in relation to surface phagocytosis (Foley *et al.*, 1960; Foley and Wood, 1960) and concluded that the M protein and the hyaluronic acid capsule are both important factors in phagocytosis. They found that a very definite quantitative relationship existed on the one hand between the size of the capsule and amount of M protein and, on the other, with susceptibility of a strain to phagocytosis and its degree of virulence. Two strains possessing equivalent amounts of M protein nonetheless had a different susceptibility to phagocytosis in that the one with the smaller capsule was more readily phagocytized. An-

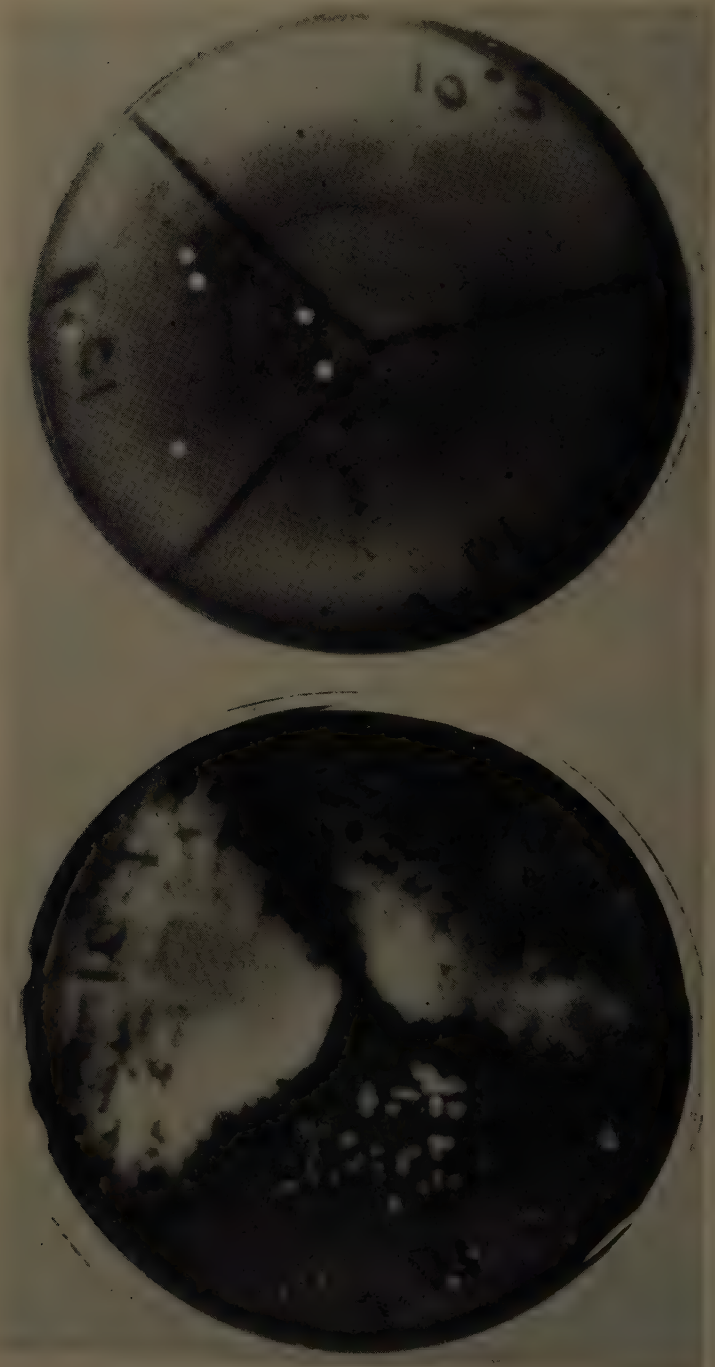


FIGURE 2.

other strain that produced no M protein had a large capsule that gave it some protection against phagocytosis. A fourth strain that had both diminished capsule and M protein content was readily phagocytized. These strains had an intraperitoneal virulence for mice or rats that was proportional to their susceptibility to phagocytosis. Although they do not emphasize the point, their results clearly show that M protein is quantitatively more important than C polysaccharide as an antiphagocytic and virulence factor.

Spread of Q Cultures in Tissues of Injected Mice; Effect of Antiserum and Removal of M Protein

We have tried to demonstrate the relationship of M protein to virulence by following the spread of the Q organisms to the organs of injected mice. The contribution of capsules was minimized, at least in the initial stages of infection in these experiments, by employing 18-hour cultures. Capsules are absent from cells in broth cultures of the Q strains that are older than 6 to 9 hours. The fate of Q organisms in mice may be followed in TABLE 5. Intraperitoneal injection of approximately 10^6 chains of each Q culture into separate groups of mice resulted in a similar picture in so far as the number of organisms that could be recovered from the spleens of these animals 1 and 3 hours after injection. In both groups, approximately 3000 to 5000 streptococcal chains could be found in the spleens at the end of 3 hours. By 5 hours, however, no viable organisms could be recovered from the spleens of the animals injected with the Q496 culture, whereas there were now 150,000 or so organisms in the spleens of the Q43x-injected animals. By the 7th hour, these numbers had increased fourfold in the spleens of the mice that received the virulent culture. Wood states that phagocytosis in the first hour of injection is pri-

TABLE 4
INDIRECT BACTERIOSTATIC TEST USING HUMAN BLOOD

	Dilution of culture					
	10^{-4}	10^{-5}	10^{-6}	10^{-4}	10^{-5}	10^{-6}
	Normal rabbit serum			Type-14 rabbit serum		
Q496, Growth on plates	+++	++	5	++	++	0
Q43x, Growth on plates	++++	+++	33	3	0	0

TABLE 5
FATE OF Q CULTURES INJECTED INTO MICE

Culture	Streptococcal chains injected	Streptococcal chains recovered from spleen at various times after injection			
		1 hr.	3 hr.	5 hr.	7 hr.
Q496	1.5×10^6	4.1×10^3	5.5×10^3	0	0
Q43x	1.0×10^6	2.2×10^3	3.2×10^3	1.5×10^5	6.0×10^5

marily by lymphocytes present in the peritoneum. In the succeeding hours, a polymorphonuclear leukocyte invasion of the peritoneum takes place.

The question might then be asked: How extensive does the multiplication and spread of the invading streptococci in the tissues of the host have to become before the animal succumbs? TABLE 6 presents data in this regard. The group of animals studied here were also given a large dose, namely 10^6 chains, of the Q43x culture. Animals were sacrificed at various periods following infection, and the number of viable chains in the spleen, liver, kidney, and heart were determined. All of the animals in the group were dead by 12 hours after infection. It is evident that the infection took rapid hold; by the third hour the organs studied had 10^4 to 10^5 organisms in them. There was a rapid multiplication, so that by six hours there had been an average increase of 100-fold in the number of chains, and by the time of death each of the organs had at least 10^8 chains. This is a massive spread indeed. These findings have been verified a number of times, and we can safely say that most mice succumbing to the infection with Q43x cultures were harboring approximately 10^9 or more organisms. We may defer a brief consideration of the question of what has actually caused the death of the animal.

TABLE 6

SPREAD OF Q43x ORGANISMS IN MOUSE TISSUES AFTER IP INJECTION OF 10^6 CHAINS

Time after injection (hours)	Streptococcal chains in			
	spleen	liver	kidney	heart
3	1.6×10^5	5.2×10^5	1.6×10^5	1.2×10^4
6	1.7×10^7	2.0×10^7	4.0×10^6	1.6×10^6
12	2.2×10^8	3.8×10^8	2.8×10^8	2.7×10^8

All animals died by the 12th hour after injection.
Values given are the average of 3 animals at each time period.

TABLE 7

EFFECT OF TRYPSIN EXPOSURE ON VIRULENCE OF Q43x CULTURE IN MICE

Experiment	Cells suspended in	Time of exposure (min.)	Streptococcal chains injected	No. died/No. injected
1	Buffer, *p*H 8	45	7.5×10^4	4/5
	trypsin, buffer	45	3.0×10^4	3/5
2	Buffer, *p*H 8	90	4.2×10^5	5/5
	trypsin, buffer	90	3.5×10^5	1/5
3	Buffer, *p*H 8	180	1.8×10^6*	5/5
	trypsin, buffer	180	1.8×10^6*	1/5

* No. of chains exposed to treatment. Counts were not made following buffer and trypsin exposure.

Let us now turn our attention to some experiments that demonstrate further the role of M protein in establishing the infection. In TABLE 7 are given the results of 3 experiments involving 10 animals in each. Five animals in each group were injected with challenge doses of Q43x streptococci that had been subjected to varying periods of exposure to trypsin. Five animals in each group served as controls and were injected with organisms that were exposed to the phosphate buffer for the same periods and under the same conditions. Trypsin is known to remove the M protein without destroying the viability of the organisms (Lancefield, 1943). It is evident that an exposure time of 45 min. was insufficient to alter significantly the death rate from the challenge. However, in experiments 2 and 3, 4 of 5 animals challenged with organisms that had been previously exposed to trypsin survived, whereas all of the control animals died. Unless this short exposure to trypsin has succeeded in affecting the streptococci in some more subtle way, other than by removing the M protein, it may be concluded that these experiments demonstrate that the ability of the Q43x culture to establish an infection is dependent upon the presence of the M protein that, as has also been seen, decreases the susceptibility of the organism to phagocytosis.

TABLE 8

THE EFFECT OF PRETREATMENT WITH TYPE-14 RABBIT ANTISERUM ON THE SPREAD OF Q43x ORGANISMS IN MICE*

Experiment	Challenge (no. of chains)	Time after challenge (hours)	No. streptococcal chains in spleen	
			Pretreatment with	
			Normal rabbit serum	Type-14 rabbit antiserum
1	3.0×10^6	1.0	4.9×10^3	1.2×10^4
		3.0	7.1×10^4	3.9×10^4
		6.5	4.1×10^6	2.2×10^5
2	2.3×10^5	2.0	1.6×10^3	0
		5.0	1.1×10^4	2.0×10^3
		8.0	2.1×10^5	8.7×10^3

* Values given are the average of 2 or 3 animals in each time period.

Further data bearing on the role of phagocytosis in the virulence of these organisms in mice is seen in TABLE 8, in which the spread of infection in animals that have been pretreated by 24 hours with type-14 immune serum is compared to control groups pretreated with normal rabbit serum. Large challenge doses were employed. In the animals given normal rabbit serum, the picture is similar to that seen earlier in TABLES 5 and 6. The animals pretreated with immune serum in the first experiment showed a slower multiplication of the injected organisms (at 3 and 6½ hours) as compared to the control group. The challenge dose in this experiment was large and an antiserum of only moderate titer was employed. Therefore, there was only a delay in the time of death and not complete protection. In the second experiment, a smaller challenge dose was given. The antiserum-treated group showed a markedly decreased multiplication of injected organisms. The additional animals that were not sacrificed for counts in this group all survived. It is a reasonable assumption that the decreased and delayed spread of organisms in the partially protected group was the result of enhanced phagocytosis that occurs in the presence of immune serum. Nonetheless, it is evident that immune serum was not effective in preventing spread to the spleen but rather to limit the rate of multiplication of organisms that had become lodged there by the third hour after injection.

It was of interest to determine also the extent of multiplication and spread of organisms in the tissues of mice that were given lethal doses of the avirulent Q496 culture. These results are presented in TABLE 9. It is seen that the tissues of mice that were sacrificed just prior to death do not show quite as high a bacterial count as was found in animals succumbing to infection with the Q43x organisms. This might be a reflection of a higher metabolic potential of this strain. It appears that the Q496 organism, if given in sufficient numbers so as to overwhelm the phagocytic powers of the host, can ultimately produce death when the number of chains has reached the order of that found in the terminal stages of infection with the virulent strain. It is reasonable to believe, therefore, that the extracellular products elaborated by the avirulent organism *in vivo* are as capable of producing death as those of the virulent strain. This tentative inference is of importance in a consideration of the factors that underlie virulence. It suggests that the essential differences in virulence of strains rests not in their capacity to form exotoxins *in vivo*, which probably brings about the death of the animals, but in the ability to survive and multiply in the host to a point that allows a sufficient production of exotoxins.

TABLE 9
NUMBERS OF ORGANISMS IN TISSUES OF MICE GIVEN A MASSIVE INOCULUM OF Q496 CULTURE*

Time after injection	Streptococcal chains in			
	spleen	liver	kidney	heart
24 hrs.	1.23×10^7	1.66×10^7	1.66×10^6	1.78×10^7
24 hrs.	1.57×10^7	5.65×10^6	1.25×10^4	1.40×10^6

* Given 1.15×10^9 chains by intraperitoneal injection.
Animals sacrificed were almost terminal.

Extracellular Products of Streptococci

An assessment of the role that the known products of the streptococci individually and collectively play in bringing about death of the infected mice or of their role in the symptomatology and pathology in human infections has not yet been forthcoming. Nonetheless, considerable work has been done with the various components, and newer findings have been presented almost yearly on one or another of the toxins or enzymes elaborated by this energetic group of organisms. Although definitive proof is lacking, it is not unlikely that streptokinase and hyaluronidase can contribute to the rapid extension seen with streptococcal cellulitis (MacLeod, 1958). The hemolysins, streptolysin O and S, are potent toxins that in small amounts can cause death of animals and bring about cardiac arrest of isolated perfused amphibian and mammalian hearts (Bernheimer, 1954). Streptolysin O has also been reported to have a leukocidal effect (Todd, 1942). The DPNase elaborated by certain types of streptococci alluded to earlier seems to be associated with the ability of such types to bring about a rapid immobilization and lysis of leukocytes that have ingested such organisms. The role of streptococcal proteinase, of the several serologically distinguishable DNases, and of RNase in the process of streptococcal infection is even more undefined.

In recent years Watson (1954) and his co-workers have provided some interesting insights into host-parasite factors in group A streptococcal infections by attempting to fractionate and define the properties of extracts of streptococcal lesions that were made by the intracutaneous injection of large numbers of streptococci in multiple sites of American-Dutch rabbits. Such lesion extracts, when injected into the same strain of rabbit, caused increased susceptibility of these animals to subsequent injections of gram-negative bacterial toxins, of streptolysin O, and of erythrogenic toxin. In sufficient concentrations they were able to cause focal necrosis in the heart and liver and some kidney pathology following single injections. Extensive areas of muscle necrosis were also observed. In addition, lesion extracts were capable of elevating temperature in rabbits. In an article that appeared in the last month, Watson (1960) presented convincing evidence that the factor in streptococcal lesion extract that causes these phenomena is identical with the erythrogenic toxin. Aside from the importance of this finding, the approach that he and his co-workers have used in focusing attention on the host-parasite relationship in studying host modification by streptococcal products produced *in vivo* is an important one.

In a preliminary way, we have approached this aspect of the problem recently. If it is reasoned that the animals observed to die with streptococcal infections were harboring of the order of 10^9 chains and that death was in all likelihood due to the products that this number of organisms elaborated *in vivo*, it would be of interest to see whether filtrates of broth from the same number of organisms grown *in vitro* would have a lethal or any other effect in animals.

As seen in the upper part of TABLE 10, sterile filtrates of supplemented brain heart infusion broth, in which the Q496 or Q43x cultures were grown to a concentration of about 10^8 chains, did not have any toxicity for mice when injected by intraperitoneal route. This was also true of broths supplemented with blood during the period of incubation. More recently the latter has been confirmed with filtrates of blood-supplemented brain heart infusion in which the organisms have grown up to 10^7 chains. The tentative conclusion would be that the *in vitro* elaborations of a number of organisms of the same order that caused death *in vivo* are either qualitatively different or their quantitative difference is sufficient to produce death.

In the lower part of TABLE 10 are listed the organ filtrates prepared from animals that had been injected with the Q43x culture and were sacrificed when they appeared quite ill. These are not toxic for mice when given in aliquots derived from portions of organs that harbored the number of the streptococcal chains specified. These are, however, only preliminary results since it would have been more convincing had we injected filtrates of organisms that had as high a concentration of chains per aliquot injected as was found to be present in the mice that succumb to streptococcal infections, as shown in TABLE 6. Nonetheless, there have been some interesting recent developments in this type of experiment. A corresponding group of animals, shown in the lower part of TABLE 10, that received aliquots of the same sterile organ filtrates indicated subsequently, but on the same day, also received an intraperitoneal injection of approximately 100,000 chains of Q496, an amount which would usually not kill any of the animals injected. We have seen in TABLE 5 that animals injected with even larger numbers of the Q496 culture no longer harbor any viable organisms after 6 hours. For a period of several days, there was no obvious difference between the animals that had received only the sterile organ filtrates as indicated in TABLE 10 and the corresponding groups which had received the Q496 culture in addition to these filtrates. However, subsequently several animals died in the Q496-injected groups. The organs of some of the animals that had died were recovered and a quantitative estimate of any organisms present was made. There were large numbers of hemolytic streptococci throughout the spleen and liver. This was an interesting finding, since apparently the animals harbored the Q496 organisms in a latent condition for many days. In some of them, apparently something tipped the balance and the Q496 culture began to multiply and disseminate. The prior injection of filtrates of organs that had been harboring streptococci must have modified the host response so that organisms that are known to be essentially avirulent and rapidly killed were able to exist for relatively long periods in a viable state in the tissues of the animals so pretreated. This is a provocative concept and may fit in with the ability of streptococcal organisms to persist in the throats of humans after the acute episodes of streptoccal infection despite the fact that in a number of instances the cultures isolated from persons so convalescing are nontypable. Aside from being avirulent for mice, presumably such organisms would be readily phagocytized by the host, yet they persist. There must be

TABLE 10
LACK OF TOXICITY OF STERILE CULTURE AND ORGAN FILTRATES FOR MICE

Filtrates injected*	Media employed	Streptococcal chains corresponding to aliquots given
Culture		
Q496	BHI, glucose, salts	1.1×10^8
Q43x	BHI, glucose, salts	4.7×10^7
Q43x	BHI, glucose, salts, 5% blood	1.8×10^6
Organs of Q43x injected mice		
Spleen		1.0×10^7
Liver		1.4×10^7
Kidney		4.0×10^6
Heart		1.5×10^5

* Given by intraperitoneal route.

some kind of an altered host response following the acute phase of the streptococcal pharyngitis in such instances. In his recent article, Watson (1960) cites a report by Raska and Rotta (1956) who confirmed the earlier studies of Watson and his collaborators relative to streptococcal lesion extract, and additionally "they showed that in toxin-treated animals, group A streptococci given intranasally persist in the lung, myocardia, and kidney. Normal animals given streptococci by the same route failed to become infected."

Studies of Cell Walls of Q Cultures

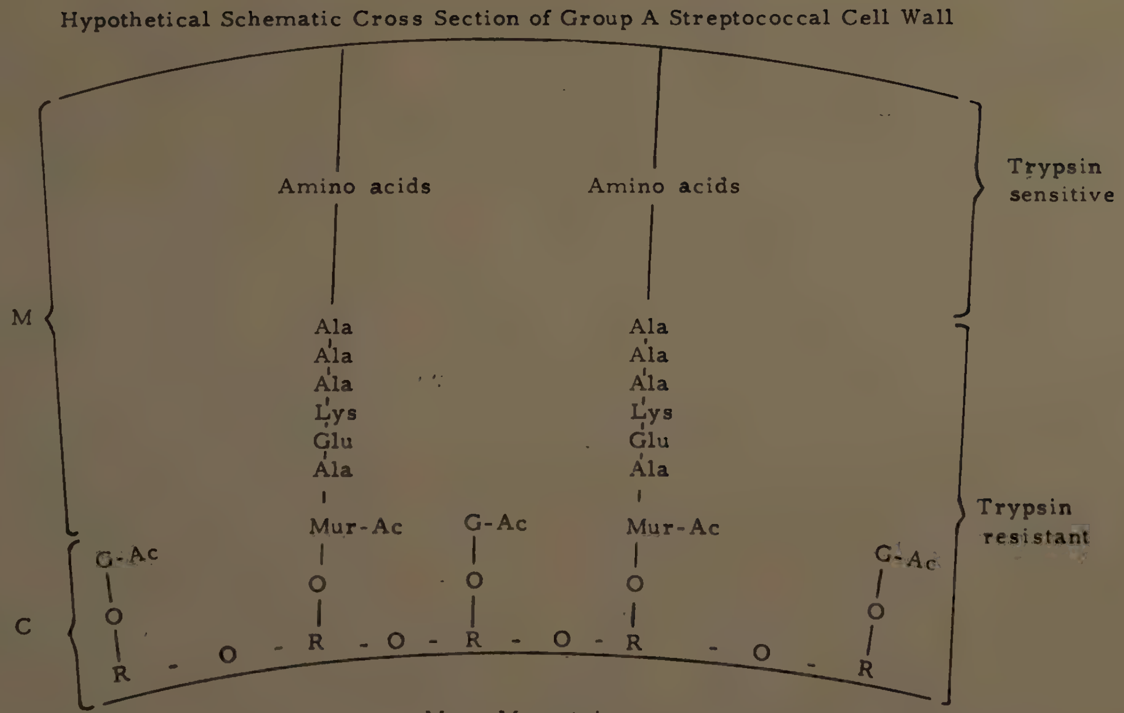
I now leave these interesting considerations, because our data along these lines are yet too preliminary to allow much more than speculation, and continue the thread of my story concerning the biochemical properties of streptococci that are the primary determinants of virulence, namely the surface components. The data of Wood and others has already been cited, indicating the role of the hyaluronic capsule. We have been more concerned with the role of the M protein. TABLE 11 taken from Leedom and Barkulis (1959) gives some indication of the quantitative difference in the M protein content of the cell walls of the virulent Q43x culture, as compared to the Q496. Estimates by ring test are, of course, crude, but unfortunately we do not have good methods of quantitating M protein. A more quantitative meaning to the data in this table can be given by indicating that the antisera that were used for these ring tests would give a positive test with purified M protein at a concentration of 5 μg./ml. Therefore it can be said that the extract from 5.35 mg. of dried cell walls of the Q496 contain less than 5 μg. of type-14 M protein, whereas 4.20 mg. of the cell walls of the Q43x strain must have contained at least 400 μg. of material that could be serologically identified as typable M protein. This represents approximately 10 per cent of the cell walls.

TABLE 11
TYPE-14 SEROLOGIC REACTIVITY OF EXTRACTS OF CELL WALLS OF Q CULTURES*

Culture	Cell walls extracted (mg. dry wt.)	Highest dilution of extract with serologic reactivity†
Q496	5.35	0
Q43x	4.20	1:80

* Reproduced by permission of the *Journal of Bacteriology.*
† Ring tests with type-14 antiserum.

Before presenting quantitative analyses of the amino acid composition of the cell walls from the Q strains, it might be helpful to illustrate in schematic fashion a simplified version of what we know and suspect of the constitution of group A streptococcal cell walls. A drawing depicting a cross-section of such a cell wall is shown in FIGURE 3. I emphasize the qualification that this figure represents an uncomplicated version that is consistent with the known facts. Structural fidelity is not implied. A detailed consideration of the evidence from our and other studies on which this picture is based would constitute more than a lecture. I can cover only some of the high points of our present concepts. To



M = M protein
C = C polysaccharide
Mur = Muramic acid
G = Glucosamine
Ac = Acetyl
R = Rhamnose

FIGURE 3. Hypothetical schematic cross section of group A streptococcal cell wall.

begin with L-rhamnose and D-hexosamine, the latter present both in acetylated form and in the form of acetylated muramic acid, make up the C polysaccharide of the cell wall (McCarty, 1952; Salton, 1953; Cummins and Harris, 1956; Barkulis and Jones, 1957). The basic structure of the cell walls, however, is made up of the C polysaccharide plus a peptide unit consisting of four alanine moieties, one or two of which are in the D form (unpublished results of J. A. Hayashi), one L-lysine, and one glutamic acid moiety probably present as the D-isomer by analogy with findings in cell walls of other species (Hayashi and Barkulis, 1959). The figure shows only one peptide unit connected to each acetyl-muramic acid grouping. The quantitative data available, which will be presented in succeeding tables, allow the possibility that two or more peptide units may be joined in sequence with only the most proximal unit being attached to an acetyl muramic acid moiety. Also, existing information does not rule out the possibility that the backbone, which is here shown as a straight chain rhamnose polysaccharide, may be branched in which case the acetyl hexosamine units would then be attached in some other periodic sequence to a branched rhamnose structure. Enzymes that lyse the basic streptococcal wall fabric have been described (McCarty, 1956; Krause, 1957 and 1958). Their application to the elucidation of wall structure should permit an unambiguous choice between these possibilities and indeed allow a faithful reconstruction of the actual structure.

By inference from the work of Park (1952) and of Park and Strominger (1957), the amino acids in the peptide unit probably occur in the sequence that is shown and probably are connected to the muramic acid. This core cell wall structure is made up of the C polysaccharide, and peptide units can be obtained simply by isolating cell walls by the conventional methods and exposing them to trypsin. Schwab and Cromartie (1957, 1960) have shown that a macromolecular complex consisting of C polysaccharide and a peptide can be recovered from 1 hour sonicates of group A streptococci and produces chronic nodular lesions when injected into the dermal connective tissue of rabbits. The possibility that this phenomenon is related to the pathogenesis of rheumatic fever bears investigation. If a relationship is shown, it will implicate cell wall components in a biological role hitherto unsuspected.

The unmodified cell wall is 40 per cent heavier than the structure that makes up the basic fabric of the cell wall. The remaining 40 per cent, we believe, probably takes origin from the end of the defined peptide units and is made up of 13 other amino acids. This outer portion moiety of the cell wall, as indicated earlier, can be removed by treatment with trypsin and, at least in part, makes up the M protein. In all probability the M moiety extends to and includes the peptide unit that makes up the basic cell wall structure.

These considerations are more concretely documented in TABLE 12, which lists the quantitative composition of the nitrogen-containing components of the cell walls of the Q cultures after trypsin exposure. We see that the basic cell wall structure for both cultures has an identical composition. It is probable that these constituents are arranged in the same structural pattern in the cell walls of these two cultures. In TABLE 13 is listed the complete amino acid and amino sugar composition of the cell walls of these two organisms. These determinations, taken from Tepper *et al.* (1960), were made on walls that were previously hydrolyzed with acid and the hydrolysis products then determined by chromatography on Dowex 50 columns following the method of Moore and Stein (1954). With the possible exception of valine and isoleucine, the amount of variation that is seen in the component amino acids would seem to be within experimental error. The somewhat greater differences seen with valine and isoleucine probably represent some degradation during acid hydrolysis. We prefer the interpretation that there is no significant qualitative or quantitative difference in the composition of the cell walls of the Q496 organisms as compared to the Q43x. Nevertheless, a serologically distinguishable M protein is obtained by extraction of the Q43x organisms but not of the Q496.

TABLE 12
PRINCIPAL NITROGEN-CONTAINING COMPONENTS OF CELL WALLS OF Q CULTURES AFTER TRYPSIN EXPOSURE

	Micromoles per mg. nitrogen		Approximate mole ratios
	Q496	Q43x	
Glutamic acid	2.91	2.90	1.0
Lysine	2.70	2.88	1.0
Alanine	9.57	10.40	3.5
Glucosamine	4.86	4.83	2.0
Muramic acid	0.79	0.84	

TABLE 13
AMINO ACID AND AMINO SUGAR COMPOSITION OF CELL WALLS OF Q CULTURES

	Micromoles per mg. nitrogen	
	Q496	Q43x
Glutamic acid	6.03	6.22
Lysine	4.35	4.50
Alanine	13.40	13.20
Glucosamine	4.86	4.83
Muramic acid	0.85	0.88
Aspartic acid	3.35	3.01
Threonine	1.78	1.43
Serine	1.67	1.32
Glycine	2.05	1.78
Valine	1.43	0.93
Isoleucine	1.12	0.73
Leucine	2.02	1.74
Tyrosine	0.82	0.64
Phenylalanine	0.67	0.59
Arginine	1.25	1.02
Methionine	0.46	0.30
Proline	1.33	1.11
Ammonia	9.39	6.76

It was of interest to see if there was any significant difference in the protein that was extracted and purified from the cell walls of the two strains, using the conditions that have been previously specified by Lancefield and Perlmann (1952) for preparing M protein (Barkulis and Jones, 1957). This procedure was carried out in parallel on the cell walls of both cultures, and the proteins obtained were hydrolyzed and the quantitative content of the various amino acids determined. These findings are summarized in TABLE 14. It is apparent that the same amino acids find representation in both proteins and that there are no striking differences in the content of any one amino acid in the two proteins. There are obviously some differences, which are most pronounced in the case of lysine, aspartic acid, threonine, and glycine. However, as evidenced by the ammonia values given, there was certainly some loss of amino acids during the process of hydrolysis. We know that lysine tends to be fairly labile during hydrolysis. We feel these findings indicate that there is no essential difference in the amino acid composition of the Q496 M protein with the corresponding M protein associated of the Q43x organisms. Consequently, we are left with the conclusion that the basic differences in the serologic properties of the outer protein layer of the cell walls of these two organisms must be based on a difference in the sequence of the respective amino acids.

TABLE 14
AMINO ACID COMPOSITION OF M PROTEIN OF Q43x CELL WALLS AND OF CORRESPONDING PROTEIN OF Q496 CELL WALLS

Amino acid	Micromoles per mg. nitrogen	
	Q496 protein*	Q43x M protein
Glutamic acid	9.18	9.53
Lysine	3.95	5.65
Alanine	4.59	4.02
Aspartic acid	4.82	5.99
Threonine	1.91	2.57
Serine	2.22	2.57
Glycine	2.82	2.07
Valine	1.36	1.21
Isoleucine	0.78	0.71
Leucine	3.97	3.92
Tyrosine	0.84	0.56
Phenylalanine	0.57	0.47
Arginine	1.34	1.34
Methionine	0.28	0.38
Proline	0.58	0.28
Ammonia	6.87	7.42

* Prepared from Q496 cell walls in the same manner as was M protein from Q43x cell walls.

Summary

We may say that a primary determinant of the virulence of these two streptococcal cultures is the structure of the outer protein layer of the respective cell walls. This difference in structure of the streptococcal surfaces expresses itself biologically by a varying susceptibility to phagocytosis. The protein layer of the cell walls is made up of 16 amino acids, which are found in equal amounts in the cell walls of both organisms. For some reason, the structure associated with type specificity either repels leukocytes or fails to attract them. While this is a primary determinant of the virulence of the two cultures, in all probability the biological effects of streptococcal infection (death of mice in our studies), probably including the clinical entities recognized in human infections, is due to the exotoxins produced by these organisms in the course of successful lodgment and multiplication. There is also some evidence, however, that these streptococcal products may modify the host response in that the tissues of a previously infected host may permit the presence of viable organisms that are no longer highly virulent and *in vitro* can be readily phagocytized. The continued presence of such organisms would allow a constant production and dissemination of toxins, causing sensitization of tissues. Subsequent infections with other types of group A streptococcus might then initiate clinical conditions such as nephritis and rheumatic fever, which are thought to have a sensitizing or immunologic factor in their pathogenesis.

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DNA SYNTHESIS, RESPIRATION, AND VIRULENCE IN PNEUMOCOCCI*

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It has been recognized for many years that virulence of pneumococci depends upon the presence of a specific capsular polysaccharide (SSS) on the surface of the cells (Burrows, 1951; Dubos, 1954). Strains of virulent pneumococci differ qualitatively with reference to the chemical composition of the capsular SSS and quantitatively in the extent of capsule synthesized. The chemical nature and quantity of capsular material present in any one strain is genetically controlled, but specific environmental conditions can alter its expression (Dubos, 1954). Although encapsulated pneumococci resist phagocytosis *in vivo*, isolated capsule is nontoxic when injected into animals, and it is not clear precisely how the polysaccharide interacts with phagocytes in susceptible hosts. Presumably, the inhibition of phagocytosis would permit the organism to proliferate extensively and elaborate specific toxins or interfere with essential functions of the host by competing for essential nutrients. Nevertheless, certain results have been indicative of the fact that components of the capsule, or the capsule itself, possess toxic properties. For example, Alonzo and Nungester (1956) demonstrated that several moieties of the type I pneumococcal capsule (glucosamine, particularly) inhibited glycolysis of rat leucocytes. Macleod and Krauss (1950) were able to correlate the amount of SSS produced *in vitro* by various pneumococcal types with the virulence of such types in mice.

The severe intoxication observed in *Pneumococcus* infections in man and animals has been suggestive of the formation of some toxin other than SSS by this pathogen. As early as 1891 Bonome isolated a substance from a pneumococcal culture that was lethal for rabbits. Cole (1912) and Rosenow (1912) were able to elicit severe and fatal toxic reactions with saline extracts and bile solutions of pneumococci. Some of the intracellular substances that were released by the autolysis of the organisms were eventually characterized as proteins that were found capable of hemolyzing erythrocytes of several animal species (Cole, 1912). Another intracellular toxic substance of unidentified chemical nature, the "purpora-producing principle," was isolated by Julianelle and Reimann in 1926. This principle produced a pathological condition in animals that manifested itself as a dark blue discoloration brought about by hemorrhaging in various parts of the body. This condition has also been observed in fatal infections of pneumonia in man (White, 1938). Other toxic components released by autolyzing pneumococci have included leucocidins (Oram, 1934) and necrotizing substances (Parker, 1928).

Although preparations containing the just cited components increase the virulence of pneumococci when they are injected simultaneously with the bacteria, there is evidence to support the fact that some of the toxicity of pneumo-

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cocci *in vivo* can be traced directly to metabolic activities of the organisms during the spreading phase of the disease rather than to specific toxins released as a result of cellular disintegrations (Dubos, 1954). For example, most of the manifestations of acute toxicity in pneumococcal lobar pneumonia disappear in a few hours after treatment with an effective antibiotic despite the presence of lesions and other typical pathological characteristics. If toxins are present to any significant degree, disease conditions should prevail for some time after the onset of therapy-inhibiting bacterial growth. If it is assumed that the antibiotic has no direct antitoxic properties, it can be concluded that a reaction carried out by the multiplying pathogen is detrimental to host metabolism and is inhibited by the therapeutic agent. Some possible reactions carried out by the actively metabolizing pathogen that have been suggested as toxic for the host are the conversion of hemoglobin to methemoglobin, which would reduce the oxygen-carrying capacity of the blood (Neill, 1925), the production of toxic levels of H_2O_2 during glycolysis *in vivo* (Morgan and Neill, 1924), and the production of hyaluronidase (Humphrey, 1944). Active metabolism of the pathogen *in vivo* undoubtedly requires proper environmental conditions that may be found in susceptible hosts but not in resistant ones. That the potential pathogenic properties of pneumococci can be controlled by the physicochemical characteristics of the *in vivo* environment has been suggested by a number of investigators (Dubos, 1954 and White, 1938). Their conclusions have been supported by such findings as those of Bernheimer (1953) who observed that the polysaccharide capsule of type III pneumococci was most readily formed and least soluble from *p*H 6.3 to 6.7. Thus the capsular material of pneumococci would be most likely to remain in the insoluble form under relatively acidic conditions such as in inflammatory areas where intense glycolytic activity occurs (Menkin, 1950). Also, the resistance of mice to the intraperitoneal injection of type I pneumococci was found to be markedly affected by the type of diet on which the animals were maintained (Hitchings and Falco, 1946). The active principle in the diet turned out to be Mn^{++}. This was probably the first observation that concerned a specific, naturally occurring environmental factor that enhanced the virulence of pneumococci in susceptible hosts. Another environmental factor that at first appeared to be important in enhancing the virulence of pneumococci in susceptible hosts was uncovered by Griffith (1928). He was able to recover virulent encapsulated pneumococci from mice succumbing to infection when these animals were injected with a mixture of nonencapsulated, avirulent bacteria and heat-killed encapsulated pneumococci of the same type. This transformation of an avirulent population to a virulent one *in vivo* was found to be controlled by the genetic material (deoxyribonucleic acid) of the heat-killed encapsulated pneumococci (Avery *et al.*, 1944). However, the significance of this transformation has been questioned by several investigators, notably Austrian (1952) who pointed out that highly artificial conditions were required for the success of the phenomenon *in vivo*. In addition, it was impossible to demonstrate transformation in the respiratory tract, the natural habitat of pneumococci.

Such naturally occurring and variable factors controlling the outcome of host-parasite relationships have been uncovered relatively infrequently in the past. However, more recently, studies by several investigators of the factors responsible for the selection and proliferation of spontaneously arising virulent mutant cells in initially avirulent populations *in vitro* have revealed additional factors that may be significant to the understanding of the factors that contribute to the selective survival and multiplication of virulent types of a pathogen *in vivo*. Under most artificial conditions, that is, in lifeless laboratory media, it is well established that populations of virulent bacteria tend to undergo gradual population changes involving the establishment of spontaneously arising, less virulent mutants. In contrast, in susceptible hosts such changes are in the opposite direction, that is, more virulent variants attain a higher selective value than their less virulent progenitors or sister cells. Braun and Whallon (1954) and Firshein and Braun (1958) have described the ability of enzymatic digests of DNA derived either from bacterial or mammalian sources to stimulate bacterial population changes *in vitro*, resulting in the establishment of spontaneously arising virulent mutant cells in initially avirulent or predominantly avirulent populations. It was shown that highly polymerized DNA treated with the enzyme DNase promoted rapid population changes from R (avirulent) to S (virulent) in *Brucella abortus* cultures and in *Diplococcus pneumoniae* cultures. Particularly in the latter, the presence of other nucleic acid breakdown products were required for optimal effects in conjunction with DNA + DNase (all 8 of the naturally occurring deoxynucleosides and deoxynucleotides, and the 4

TABLE 1

POPULATION CHANGES IN AGING BROTH CULTURES INITIATED WITH AVIRULENT OR PREDOMINANTLY AVIRULENT INOCULA OF PNEUMOCOCCAL STRAINS OR OF *BRUCELLA* STRAINS IN THE PRESENCE OF ENZYMATIC DNA DIGESTS, DEOXYNUCLEOSIDES, DEOXYNUCLEOTIDES, AND NUCLEOSIDE-DI-PHOSPHATES

	DNA + DNase*	Deoxynucleosides, deoxynucleotides, nucleoside-di-phosphates†	Percentage of virulent (S) cells in brain-heart-infusion blood broth (pneumococci) or buffered beef-extract broth (*Brucella*)	
			48 hours	5 days
Pneumococcal strains				
SVI (1%) + RI 192 (99%)	+	+	95	
(both of type I)	+	−	4	
	−	−	0	
D39S (1%) + R36A (99%)	+	+	65	
(both of type II)	+	−	10	
	−	−	0	
RV497 (100%) (originated from	+	+	70	
type V)	+	−	20	
	−	−	8	
Brucella strains				
19 (100%) (mucoid)	+	−		60
	−	−		1

* One hundred and fifty μg./ml. + 25 μg./ml.

† Eight hundred, 800, 100 μg./ml., respectively.

nucleoside-di-phosphates derived from RNA (TABLE 1). The required DNA digest factor was found to be nonidentical with known breakdown products of DNA (Braun, 1956). Although the same end result was obtained, that is, a selection of virulent cells in initially avirulent or predominantly avirulent populations, the mechanism responsible for the R → S population changes appeared to be completely different for *Brucella* and *Pneumococcus*. Whereas virulent *Brucella* mutants were selected because of an inhibition of avirulent cells without any appreciable effect on virulent cells, selection in *Pneumococcus* cultures resulted from a striking selective stimulation of the growth of virulent cells in the presence of DNA digests without any marked effect on avirulent pneumococci (FIGURE 1).

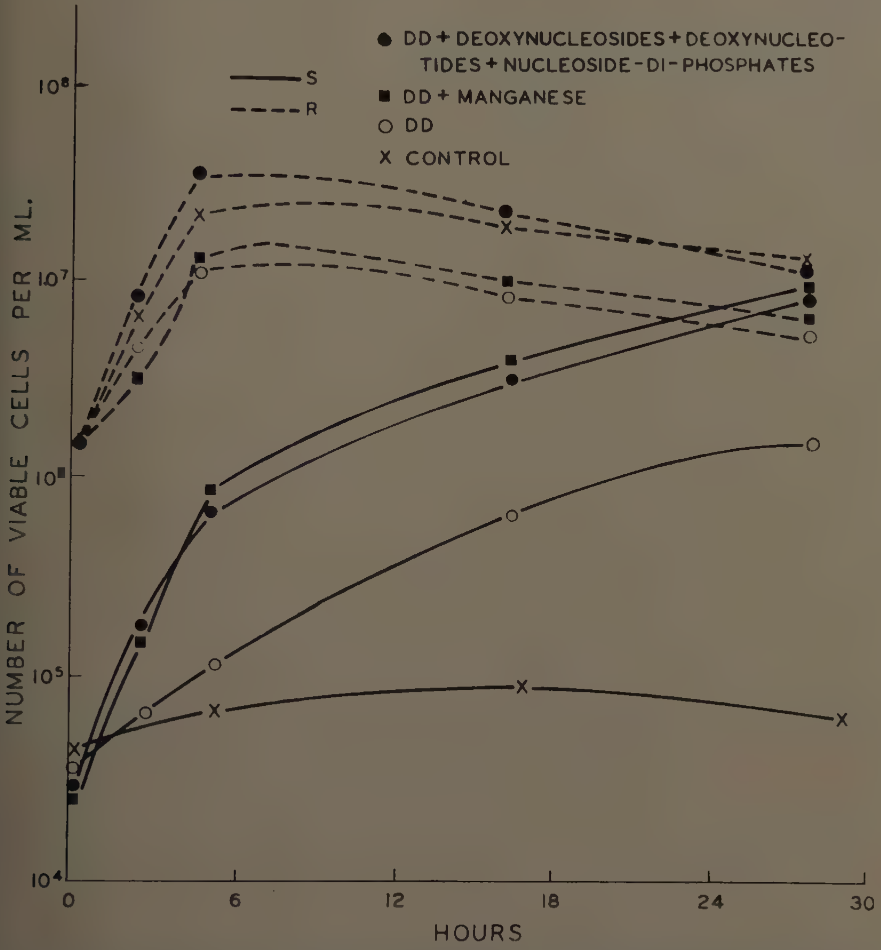

FIGURE 1. Viable counts of S and R type I pneumococci undergoing population changes in the presence and absence of various combinations of enzymatic DNA digests, Mn^{++}, deoxynucleosides, deoxynucleotides, and nucleoside-di-phosphates in brain-heart-infusion blood broth. Inoculum: 1 per cent SVI + 99 per cent RI 192.

In a search for factors other than the nucleosides and nucleotides that may enhance the activity of enzymatic DNA digests in the selection of encapsulated S cells, it was found that Mn^{++}, but not other divalent cations, played a significant role. In the absence of this ion, no selective effects were observed despite the presence of the digest. Both components were necessary for optimum selective activity in the absence of the nucleoside derivatives described above, but under the proper cultural conditions and with the proper strains Mn^{++} alone could enhance R → S population changes and stimulate the growth of virulent pneumococci in a manner similar to that observed with DNA digests plus the nucleoside supplements (Firshein and Braun, 1960). Purine and pyrimidine mixtures were also found capable of stimulating growth of S cells and population changes in initially predominantly R populations provided DNA + DNase (DD) and Mn^{++} were present. Such mixtures are unable to produce any effects in the absence of Mn^{++}.

These results suggested an involvement of altered or enhanced nucleic acid metabolism of those pneumococci that displayed altered growth rates in the presence of the supplements just cited. Therefore resting cell experiments were initiated in which DNA synthesis of virulent and avirulent strains of type I pneumococci was measured in the presence and absence of the nucleic acid breakdown products. The results of these experiments revealed that when suspensions of S cells in certain basal solutions were augmented with the extracellular supplements (DD + deoxynucleosides + deoxynucleotides + nucleoside-di-phosphates), a significant increase in the intracellular DNA level occurred within a 30-min. incubation period. In contrast, such increases were not observed in unsupplemented suspensions of virulent cells or in avirulent cell suspensions that contained all of the nucleic acid breakdown products.

The stimulation of growth and DNA synthesis of encapsulated virulent pneumococci *in vitro* under the influence of the DNA digest factor and other supplements were found not to be restricted to *in vitro* events. W. Braun *et al.* (1957) demonstrated that DNA-breakdown products exerted a profound effect on the virulence of pneumococci injected into mice. A single subcutaneous injection of enzyme-treated DNA 24 hours prior to, or at the same time as, intraperitoneal injection with encapsulated virulent pneumococci resulted in a substantial decrease in the survival time and LD_{50}. In addition, viable cell counts of peritoneal washings revealed a striking stimulation of the growth of S cells when mice were treated with the DNA digest, whereas such increases were not observed in the untreated animals (Firshein and Braun, 1958).

These studies thus indicated that a basic difference may exist in the nucleic acid metabolism of virulent and avirulent pneumococci, and this difference may be of critical importance to events determining the outcome of the disease in a susceptible host. Therefore it became desirable to attempt to elucidate further the role of the extracellular supplements.

Quantitative comparisons were made between encapsulated (S), weakly encapsulated (I), and nonencapsulated (R) strains of types I and II pneumococci regarding their ability to utilize the supplements for enhanced DNA synthesis over a specified time period. The supplements tested were: DD, DD + deoxynucleosides + deoxynucleotides + nucleoside-di-phosphates (DD + DNS + DNT + NDP, the full supplementation system), and DNS + DNT + NDP without DD. In contrast to the one procedure employed to isolate and measure DNA in the earlier studies (Firshein and Braun, 1960), more exacting methods of analysis were employed in the more recent studies (Mores and Carter, 1949; Schneider, 1945; Schmidt and Thannhauser, 1945; Stumpf, 1947; Dische, 1955). In addition, H^3-thymidine and other C^{14}-deoxynucleosides were added to basal suspensions supplemented with enzymatic DNA digests to determine whether differences in the rate and extent of incorporation existed between the encapsulated virulent, capsule-deficient avirulent, or weakly virulent types. In addition to testing DNA synthesis, the influence of the nucleic acid breakdown products on RNA and protein syntheses also was determined. Protein was estimated with Folin's phenol reagent according to the method of Lowry *et al.* (1951) and RNA was determined by the orcinol method of Drury (1948). Also, an attempt was made to assess the specific environmental requirements, other than nucleoside derivatives and DNA digests, which were necessary for enhanced DNA synthesis in virulent pneumococci, particularly the role of glucose, casein digests, Mn^{++}, purines, and pyrimidines.

Using a type I encapsulated S strain (SVI) and an R mutant (RI 192) derived from another type I strain as a convenient model system,* and a freshly isolated I (weakly encapsulated) mutant of type II *Pneumococcus* (D 39I) with its parental virulent strain (D39S) as the other test system, the following results were obtained in basal solutions containing phosphate buffer (0.02M, *p*H 7.5), 0.1 per cent glucose, 1 per cent casein digest (casitone), and 0.0025 per cent catalase (FIGURES 2 and 3). After the first 30 min., an approximate 40 per cent increase in acid-insoluble deoxypentose (presumably DNA) occurred in virulent cells of type I pneumococci and a 20 per cent increase occurred in virulent cells of type II pneumococci suspended in solutions containing the full supplementation system (DD + DNS + DNT + NDP). When DD alone was added to such cell suspensions, a lesser increase in DNA (15 per cent and 8 per cent, respectively) was observed. In comparison to these effects with S cells, suspensions of avirulent cells derived from type I and weakly virulent cells derived from type II showed very slight or no increases when the DNA digest and other nucleoside derivatives were present. Although the actual level of enhancement varied from experiment to experiment for each virulent cell type, DNA increased in type I and type II pneumococci on the average 50 and 25 per cent respectively above the levels observed in the controls in the presence of DD + DNS + DNT + NDP. In some instances, particularly with type I pneumococci, these increases in DNA were as high as 100 per cent with no apparent comparable rise in the total number of cells. Since no stimulation of growth was observed and since all of the determinations are in terms of ml., the data must be regarded as reflecting an increase in DNA/S cell.

It was difficult to estimate the extent of growth accurately by employing the usual method of serial dilution to count the cells. This difficulty was encountered particularly with avirulent cells because they form chains in liquid media. Thus in order to determine precisely the extent of the selective stimulation of DNA synthesis in virulent pneumococci, it was necessary to seek more reliable parameters of growth. This was achieved by measuring protein and RNA syntheses in conjunction with DNA synthesis and viable counts. Two supplementation systems described earlier were analyzed in this manner in type I encapsulated S pneumococci: (1) DD + DNS + DNT + NDP and (2) DD + Mn + PP (PP = a mixture of adenine, guanine, cytosine, and thymine). To supplement these analyses, the influence of glucose and casitone in the basal solutions were also examined. The results of this extensive series of

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* Although R strains that were not directly related in the sense of having arisen recently by simple mutational events from the corresponding S strain were employed, it is believed that the events revealed with such strains are typical of those that may occur with more closely related strains. This conclusion is based on observations made with some freshly isolated I mutants of type II pneumococci that will be mentioned subsequently.

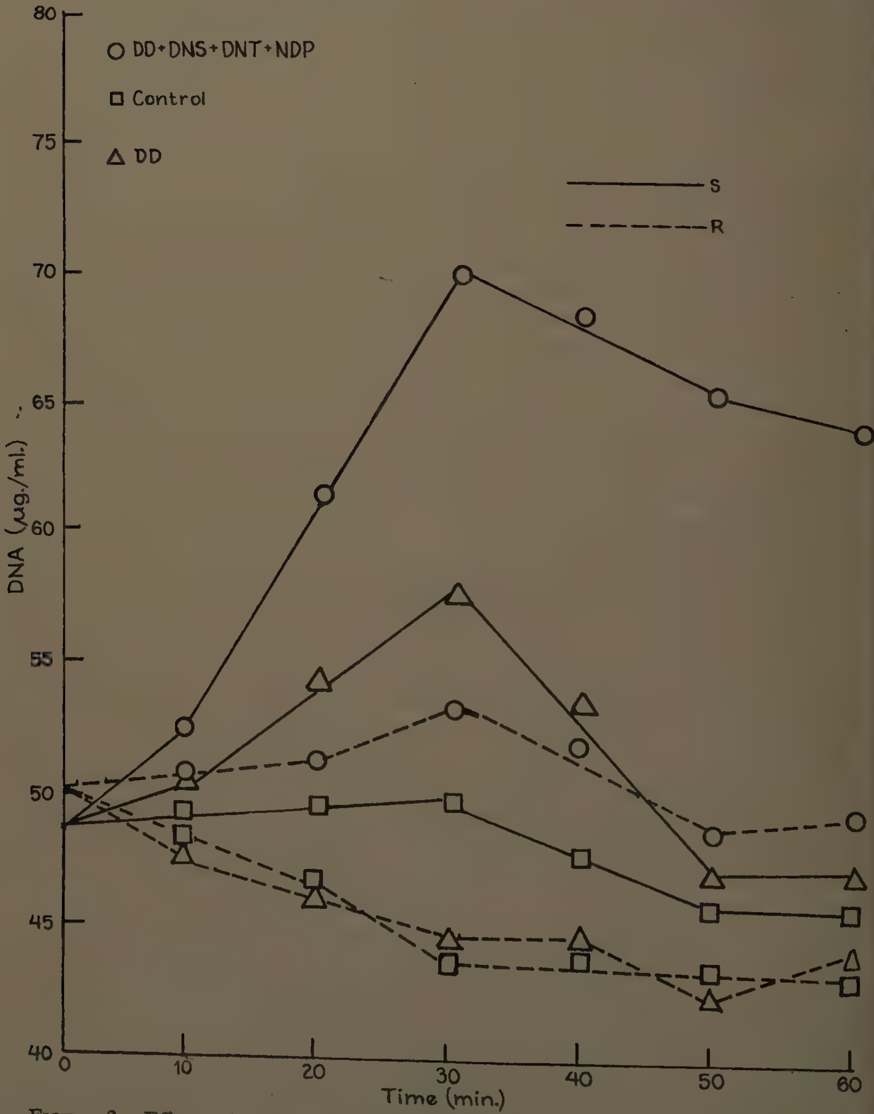

FIGURE 2. Effects of enzymatic DNA digests, deoxynucleosides, deoxynucleotides, and nucleoside-di-phosphates on levels of acid-insoluble deoxypentose (DNA) of S and R type I pneumococci in resting cell suspensions.

experiments are summarized in TABLES 2 and 3. First, it was essential to add glucose and casitone in order for maximum selective DNA synthesis to occur in virulent cells in the presence of DD + DNS + DNT + NDP. Omission of either component reduced the level of synthetic activity to one half or less than one half the maximum. In addition, in the presence of these components, enhanced RNA and protein syntheses occurred provided the nucleic acid break-

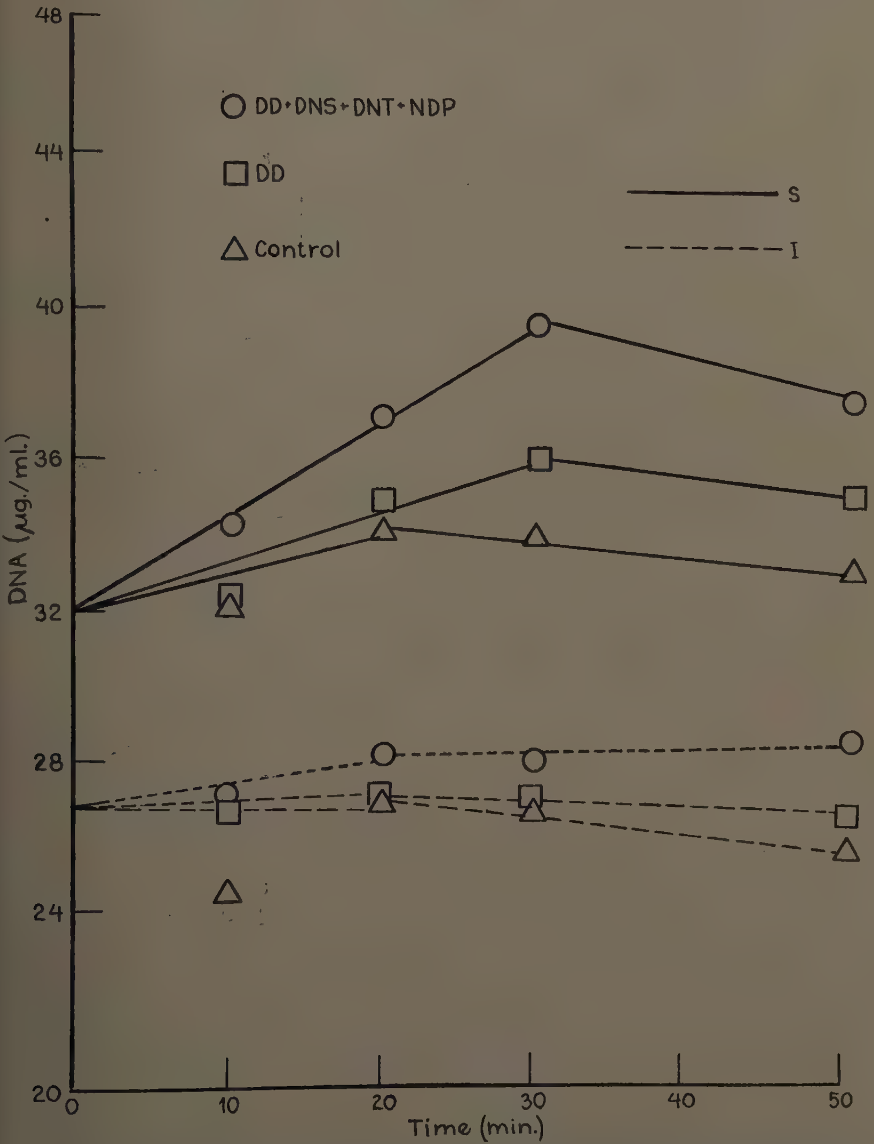

FIGURE 3. Effects of enzymatic DNA digests, deoxynucleosides, deoxynucleotides, and nucleoside-di-phosphates on levels of acid-insoluble deoxypentose (DNA) of S and I type II pneumococci in resting cell suspensions.

TABLE 2

THE EFFECTS OF 2 OPTIMUM SUPPLEMENTATION SYSTEMS ON DNA, RNA, AND PROTEIN SYNTHESES OF S AND R TYPE I PNEUMOCOCCI IN BASAL SOLUTIONS SUPPLEMENTED WITH GLUCOSE AND CASITONE

Additions*	Glucose*	Casitone*	DNA		RNA		Protein	
			S	R	S	R	S	R
			(after 30 min., μg./ml.)					
DNA + DNase + deoxynucleosides + deoxynucleotides + nucleoside-di-phosphates	+	+	39	49	136	170	438	500
	+	−	30	53	130	207	400	545
	−	+	27	52	128	190	390	540
	−	−	24	53	126	183	385	530
DNA + DNase + Mn^{++} + purines + pyrimidines	+	+	30	58	138	209	460	560
	+	−	24	56	140	210	455	565
	−	+	25	—	139	—	450	—
	−	−	26	54	131	210	420	560
Control	+	+	26	53	119	194	375	550
	+	−	24	54	106	195	370	536
	−	+	25	52	115	198	380	540
	−	−	24	56	99	193	368	540
At zero time			24	53	122	202	380	540

* Amount of supplement μg./ml.: DNA = 130, DNase = 25, deoxynucleoside mixture = 800 (200 each of deoxyadenosine, deoxyguanosine, deoxycytidine, and thymidine), deoxynucleotide mixture = 800 (200 each of the phosphorylated deoxynucleosides mentioned above), nucleoside-di-phosphates = 100 (25 each of the di-phosphates of adenosine, cytidine, guanosine, and uridine), purines and pyrimidines = 1600 (400 each of adenine, guanine, cytosine, and thymine), Mn^{++} = 36, glucose = 1000, and casitone = 10,000.

TABLE 3

PERCENTAGE DIFFERENCES IN THE EFFECTS OF 2 OPTIMUM SUPPLEMENTATION SYSTEMS ON DNA, RNA, AND PROTEIN SYNTHESES OF S AND R TYPE I PNEUMOCOCCI IN THE PRESENCE OF GLUCOSE AND CASITONE*

Additions	Glucose	Casitone	Percentage increase over zero time after 30 min.						Net increase of DNA over protein	
			DNA		RNA		Protein			
			S	R	S	R	S	R	S	R
DNA + DNase + deoxynucleosides + deoxynucleotides + nucleoside-di-phosphates	+	+	61	0	10	0	14	0	48	0
	+	−	25	0	6	0	5	1	20	0
	−	+	12	0	5	0	3	0	9	0
	−	−	0	0	3	0	1	0	0	0
DNA + DNase + Mn^{++} + purines + pyrimidines	+	+	25	9	14	3	21	4	4	0
	+	−	0	6	15	3	20	4	0	0
	−	+	4	—	14	—	18	—	0	0
	−	−	4	2	8	3	10	4	0	0
Control	+	+	8	0	0	0	0	2	8	0
	+	−	0	2	0	0	0	0	0	2
	−	+	4	0	0	0	0	0	4	0
	−	−	0	6	0	0	0	0	0	6

* Table based on data of TABLE 2.

down products were present. However, in comparison to the remarkable increases observed in DNA, the increases noted in RNA and protein syntheses were much less. DNA synthesis was also stimulated in basal solutions containing glucose, casitone, and DD + Mn + PP, but the maximum levels obtained were not as high as when the first supplement was present. The resulting increase was no longer specific for DNA, but affected RNA and protein to approximately the same extent. In the control tests, that is, in the absence of either supplement but in the presence of glucose and casitone, no significant changes in levels of protein, RNA, or DNA occurred. (It should be noted that in some subsequent experiments, higher concentrations of glucose and casitone were employed and this resulted in increases in DNA, RNA, and protein levels as shown in FIGURES 4 and 5. These increases occurred even in the absence of the nucleic acid breakdown products.)

The results just cited suggest differences in the manner by which DD + DNS + DNT + NDP and DD + Mn + PP act on S cells, the first representing a system for selective stimulation of DNA synthesis and the latter representing conditions that apparently can lead to a general stimulation of DNA, RNA, and protein syntheses. Since both of these supplements contain the enzymatic DNA digest, a logical question presents itself as to the role of this digest factor in each of the two supplementation systems. Results to be detailed in the following sections indicate that the digest may supply a cofactor for DNA synthesis.

First of all, an attempt was made to determine whether the active factor in the DNA digest provided material other than mere DNA precursors. If the digest contained a factor necessary for the control of DNA synthesis in virulent pneumococci, a critical amount of extracellular DNA might suffice to promote stimulation and in this case, the amount of extracellular DNA added could be expected to lack a quantitative relationship to the intracellular DNA formed, at least over a certain range. That this was indeed the case can be seen from the results summarized in TABLE 4. When different amounts of extracellular DNA were added to the remainder of the full supplement of DNase, DNS, DNT, and NDP, the levels of stimulation in intracellular DNA were not proportional to the amount of extracellular DNA added. Even the lowest level of extracellular DNA employed in these experiments elicited a significant enhancing response over the controls without any added DNA. Whether these differences are statistically significant must await further experimentation; nevertheless the results support the previous assumption that the factor in the DNA digest that enhances DNA synthesis by virulent pneumococci does not serve merely as a source of precursors for incorporation into the cell. This was further substantiated by experiments involving radioactive DNA prepared by growing encapsulated pneumococci of type I in the presence of C^{14}-deoxyadenosine and C^{14}-deoxyguanosine. Such DNA was digested with DNase and added to basal solutions containing the other nucleoside derivatives inoculated with S cells. The results (TABLE 5) showed conclusively that no label appeared in the intracellular DNA after a 50-min. incubation period despite the selective stimulation of DNA synthesis. Also, no label appeared even when the digest was present alone without the other nucleic acid breakdown products. If the DNA digest were supplying precursors for polymerization, definite incorpora-

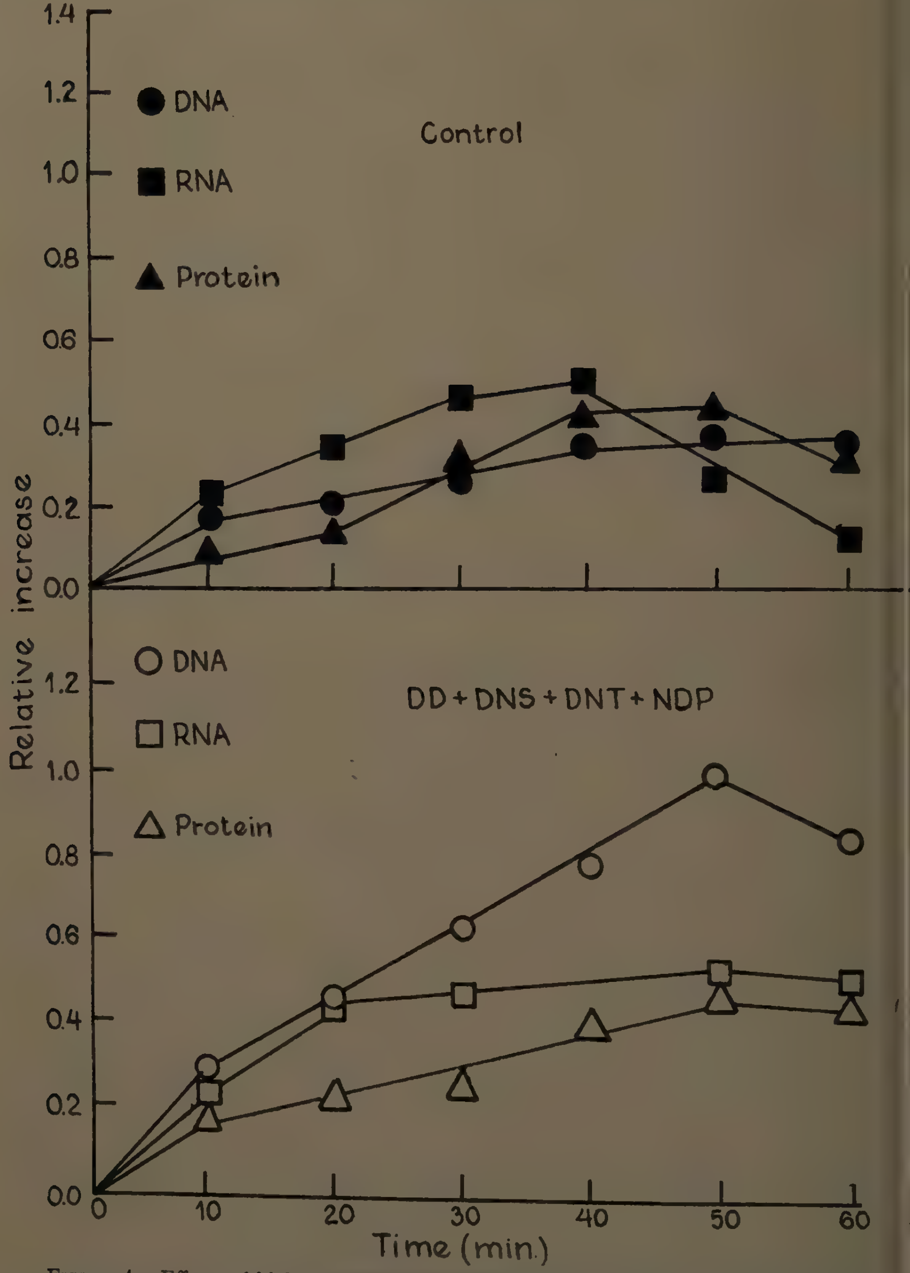

FIGURE 4. Effects of higher concentrations of glucose and casitone on DNA, RNA, and protein syntheses of S type I pneumococci in the presence and absence of enzymatic DNA digest, deoxynucleosides, deoxynucleotides, and nucleoside-di-phosphates.

tion should have been observed. The absence of such incorporation would suggest that a factor associated with a DNA breakdown product may enhance, possibly enzymatically as a coenzyme, some critical function in enhanced DNA synthesis in capsulated but not in nonencapsulated pneumococci. It is noteworthy that Lerman and Tolmach (1957) observed incorporation of extracellu-

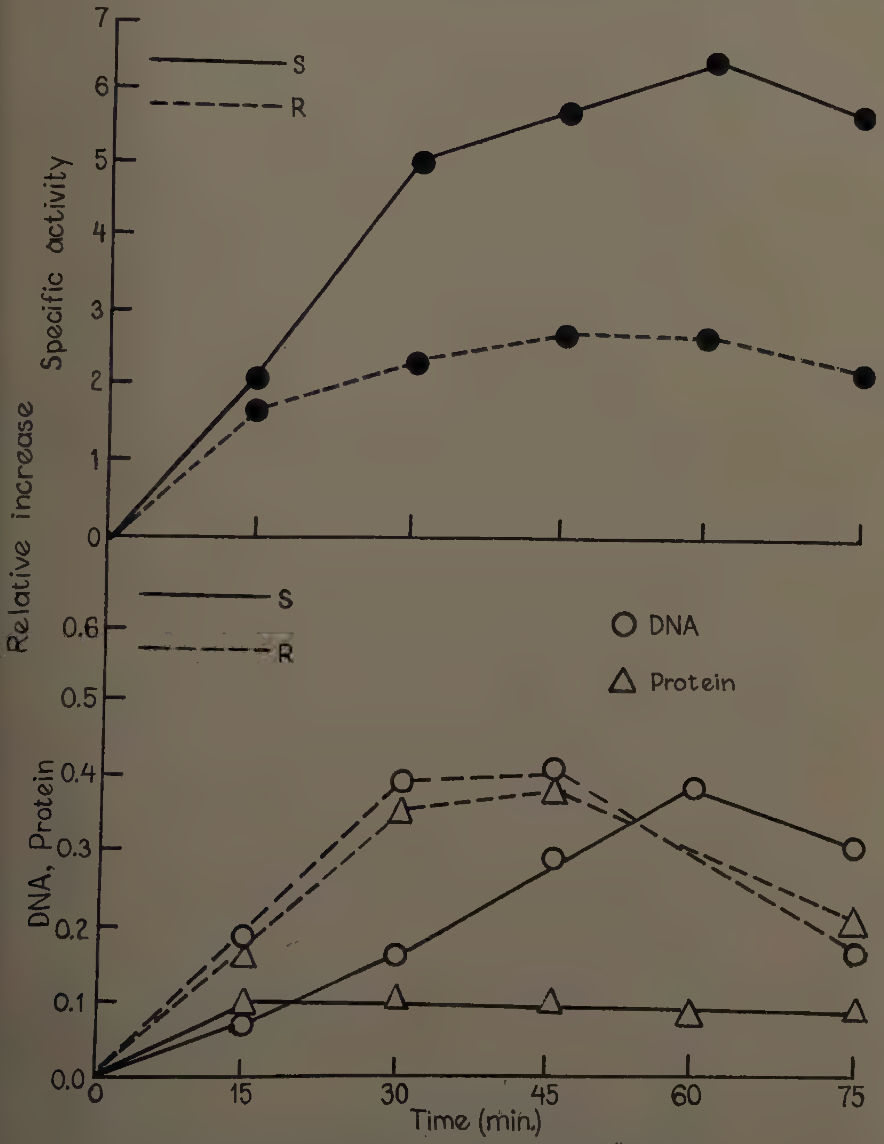

FIGURE 5. A comparison of the ability of S and R type I pneumococci to synthesize DNA and protein, and to incorporate H^3-thymidine in the presence of enzymatic DNA digests, deoxynucleosides, deoxynucleotides, and nucleoside-di-phosphates under the optimum conditions provided by the increased glucose-casitone concentrations.

lar-labeled transforming pneumococcal DNA into competent pneumococci. Therefore, it would be expected a priori that smaller molecular weight breakdown products resulting from DNase treatment of pneumococcal DNA would be incorporated into intracellular DNA. However, Lerman and Tolmach's results were obtained in growing cultures under specific environmental conditions, such as phasing of the cells by placing them at 25° C. for 30 min. prior to exposure to transforming DNA. This suggests that the cells may be permeable to such DNA only during certain phases of the growth cycle (Hotchkiss, 1954). The cells maintained in solutions described in the present experiments were not phased and were not under optimum growth conditions. Therefore it is conceivable that such cells may have metabolized the breakdown products resulting from the depolymerization of DNA in a manner different from that occurring in the transformation studies.

TABLE 4

EFFECT OF VARYING THE CONCENTRATION OF EXTRACELLULAR DNA ON LEVELS OF INTRACELLULAR DNA IN TYPE I VIRULENT CELL SUSPENSIONS IN THE PRESENCE OF A CONSTANT AMOUNT OF DNASE, DEOXYNUCLEOSIDES, DEOXYNUCLEOTIDES, AND NUCLEOSIDE-DI-PHOSPHATES

DNA concentrate added to a constant amount of DNase and the other supplements µg./ml.	After 30 min. percentage increase over zero sample			Net increase of DNA over protein	After 50 min. percentage increase over zero sample			Net increase of DNA over protein
	DNA	RNA	Protein		DNA	RNA	Protein	
1125	57	30	30	27	105	20	42	63
563	57	30	24	34	74	30	40	34
281	37	29	27	10	84	20	45	39
140	46	26	26	20	47	22	35	12
Control 1*	20	12	21	0	55	21	35	20
Control 2*	36	22	25	11	20	23	19	1
Control 3*	12	13	24	0	25	2	25	0

* Control 1 contains no DNA, control 2 contains no DNase or DNA, and control 3 is unsupplemented.

TABLE 5

THE ABILITY OF VIRULENT PNEUMOCOCCI TO INCORPORATE BREAKDOWN PRODUCTS FROM A C^{14}-DNA PREPARATION DEPOLYMERIZED WITH DNASE

C^{14}-DNA + DNase*	Deoxynucleosides deoxynucleotides nucleoside-di-phosphates	DNA	Protein	Specific activity of extracted DNA (c./min./mg.)
		(µg./ml.)		
+	+	36	435	0
+	–	28	400	0
–	+	32	425	—
–	–	26	398	—
At zero time		23	390	—

* One hundred and thirty µg./ml. + 25 µg./ml., specific activity of C^{14}-DNA before digestion is 100,150.

Considering the slight (15 per cent) but definite increases in the growth of S cells (as indicated by the elevation in total protein in the presence of DD + DNS + DNT + NDP), the concurrent rise in DNA (60 per cent) actually produced a selective increase of only 45 per cent. It soon became apparent that this selective enhancement of DNA synthesis in encapsulated pneumococci was dependent upon the amount of protein synthesized during the course of incubation. The greater the stimulation of protein synthesis, the more striking the selective rise in DNA. Similarly, a decrease in protein synthesis resulted in a corresponding decrease in the formation of DNA. It must be emphasized, however, that a selective enhancement of DNA synthesis was always observed in S cells when the full supplementation system was present in conjunction with glucose and casitone. Protein synthesis appeared to control the extent of this selective enhancement. The reasons for this variation in synthetic activity were not apparent, but they may be connected with the physiological state of the virulent cells at the time of inoculation into the basal solutions. It was very difficult to control this state accurately from experiment to experiment because of the techniques involved in preparing the inoculum, particularly centrifugation and washing procedures.

In an effort to stabilize the cells once they were inoculated, basal solutions were augmented with higher amounts of glucose and casitone (a tenfold and twofold increase, respectively). It was hoped that such enrichments would stimulate the over-all metabolism of the bacterial population, regardless of the prior metabolic state of the cells. Although some stabilization was effected by this enrichment, the fluctuations in protein synthesis were not completely eliminated. Instead, the high glucose and casitone levels frequently brought about remarkable selective increases in the DNA of virulent pneumococci when DD + DNS + DNT + NDP were added, increases that did not cease after the usual 30 min. and which elevated the DNA levels 70 per cent over the parallel net rise in protein (FIGURE 4). The over-all rise in DNA was almost 100 per cent after 50 min. In addition, the interactions that were observed between RNA and protein syntheses in the presence of DD + DNS + DNT + NDP and high concentrations of glucose and casitone strongly suggested that cell growth and multiplication were taking place (Morse and Carter, 1949; Malmgren and Heden, 1947).

Despite the previously noted absence of incorporation of labeled DNA digest compounds into S cells, the possibility remained that the differential response of S and R cells to the full supplementation system may be due to the inability of R cells to incorporate appropriate precursors as efficiently as their S relatives. Accordingly, H^3-thymidine was added to the full supplement to produce a final concentration in the enriched basal solution of 2 μc. (0.3 μg.—the ratio of radioactive thymidine to the unlabeled carrier thymidine was 1:666). After specified intervals of time, DNA and protein were extracted from duplicate samples and assayed, and an aliquot was taken from these samples for radioactive determinations. As summarized in FIGURE 5, the results showed that S cells incorporated a greater amount of H^3-thymidine/mg. of intracellular DNA than their avirulent relatives in every instance. Furthermore, the rate of incorporation of the isotope into intracellular DNA of such cells was approximately 15-fold greater than the enhanced DNA synthesis rate, suggesting that a turnover of DNA may have occurred. This DNA turnover was also observed in R-cell suspensions, but the incorporation rate was not 15 but 6 times higher than the rate of DNA synthesis. It should be mentioned that R cells were capable of synthesizing DNA from the extracellular precursors in the present experiments, although they were almost completely inert in the initial experiments (see FIGURE 2). Furthermore, the over-all rate of DNA synthesis was greater in such cells within the first 45 min. than in their virulent relatives. Since the only environmental difference between the current experiments and the initial ones involved the use of higher concentrations of glucose and casitone, it can be concluded that the ability of R cells to metabolize the nucleic acid breakdown products was controlled by high levels of certain nonnucleic acid supplements. In contrast, although high levels of glucose and casitone also enhanced the metabolism of DNA breakdown products in S cells, low concentrations of these supplements could suffice for stimulation. The important difference between the 2 cell types, however, appears to be the occurrence of a selective stimulation of DNA synthesis in virulent cells that is not detected in avirulent cells when the nucleic acid breakdown products are present. In avirulent pneumococci, DNA synthesis is proportional to the rate of protein synthesis, and this proportionality is not observed in virulent pneumococci. Thus, if protein synthesis is considered to be an indication of growth and/or multiplication, it follows that a much greater amount of DNA per cell is produced in virulent cells as compared to their avirulent relatives.

TABLE 6

INCORPORATION OF ISOTOPIC C^{14}-DEOXYNUCLEOSIDES INTO INTRACELLULAR DNA OF VIRULENT PNEUMOCOCCI IN THE PRESENCE OF THE FULL SUPPLEMENTATION SYSTEM

Addition	Isotope added*			DNA	Prot.	Percentage radioactivity incorporated into DNA
	deoxy-adenosine	deoxy-guanosine	deoxy-cytidine	(μg./ml.)		
DNA + DNase + deoxynucleosides + deoxynucleotides + nucleoside-di-phosphates	+	–	–	39	440	6.6
	–	+	–	38	444	10.0
	–	–	+	37	432	4.4
Control	–	–	–	24	400	—
At zero time				21	390	—

* Specific activities of the isotopes used/ml.: deoxyadenosine = 0.046 μc./38.6 μg., deoxyguanosine = 0.025 μc./38.6 μg., deoxycytidine = 0.021 μc./38.6 μg. The ratio of the radioactive isotope to the nonradioactive carrier in each case was 1:5.2.

The incorporation rates of 3 other labeled deoxynucleosides were also examined in S cells. The results (TABLE 6) showed differences in the rates of incorporation into DNA of the 3 isotopic deoxynucleosides in the presence of the full supplement: deoxyguanosine was almost twice as active as deoxyadenosine, which was 1½ times more active than deoxycytidine. The significance of these differences is as yet unknown.

The question arose whether the labeled precursors were incorporated into intracelluar DNA intact or were converted into other components first. These alternatives were examined by hydrolyzing the DNA synthesized after incubation in basal solutions containing C^{14}-deoxyadenosine as part of the full supplement with formic acid (Visher and Chargaff, 1948). This procedure converts DNA quantitatively into purines and pyrimidines. After chromatography and detection of the bases by ultraviolet, the spots were eluted separately and pipetted onto planchets for radioactivity determinations. Activity was detected only in the adenine eluate, indicating that deoxyadenosine was incorporated intact into the DNA molecule.

Since the previous findings had indicated that glucose enhanced DNA synthesis in virulent cells, the effects of the full supplementation system on glucose oxidation of virulent and avirulent pneumococci of type I were tested. In these studies standard respirometer techniques were employed (Umbreit *et al.*, 1951). It can be seen in FIGURE 6 that, whereas both cell types appeared to oxidize glucose to approximately the same extent in basal solutions, the incorporation of DNA digests and the nucleoside-derivative mixtures into Warburg vessels produced a striking stimulation of respiratory activity when virulent cells were present and inhibited oxygen consumption when avirulent cells were employed. In contrast, the omission of glucose from suspensions containing the nucleic acid breakdown products apparently resulted in opposite effects. Under these conditions, S cells failed to oxidize the extracellular nucleic acid breakdown products to any great extent, whereas R cells were found to exhibit definite activity in this respect. However, the latter oxidation level was still far below that noted when glucose alone was present or even when the nucleic acid breakdown products were added in conjunction with the sugar to produce inhibitory effects.

Additional studies, to be published elsewhere, have indicated that: (1) the full supplementation system definitely stimulates the oxidation of glucose in virulent pneumococci and is not extensively oxidized in the process, (2) the advantage produced by the extracellular supplements cannot be explained on the basis of nitrogen content, (3) the components of the full supplementation system that are most active in stimulating glucose oxidation are the deoxynucleotide mixture and the enzymatic DNA digest, and (4) Mn^{++} also stimulates glucose oxidation in virulent pneumococci and inhibits such respiratory activity in avirulent pneumococci under similar environmental conditions.

Discussion

The capability of virulent pneumococci to employ certain extracellular nucleic acid breakdown products for what appears to be stimulation of two different metabolic processes, DNA synthesis and respiration, and the inability of their avirulent or less virulent relatives to carry out similar activities in the presence of these supplements clearly reflects a basic metabolic difference between the two cell types. It would appear reasonable to infer that this difference could account partially for some of the properties of virulence exhibited by *Pneumococcus* in a susceptible host. As mentioned earlier, previous observations (Firshein and Braun, 1958) have demonstrated that DNA breakdown products exert a profound influence on the outcome of pneumonia in mice. If nucleic acid breakdown products, such as those described in this report, occur naturally during infections as a result of tissue and cellular disintegrations, they can be expected to stimulate the growth of the invading pathogen simultaneous with, or independent of, additional effects on the metabolic activities of the host. In either case an enhancement of the pathological condition can be expected to result. As suggested by W. Braun, 1956, these factors would be further elucidated if it could become possible to employ specific antagonists that would inhibit the pathogen's DNA synthesis and growth *in vivo* in such a therapeutic manner that the host would recover from the disease without any

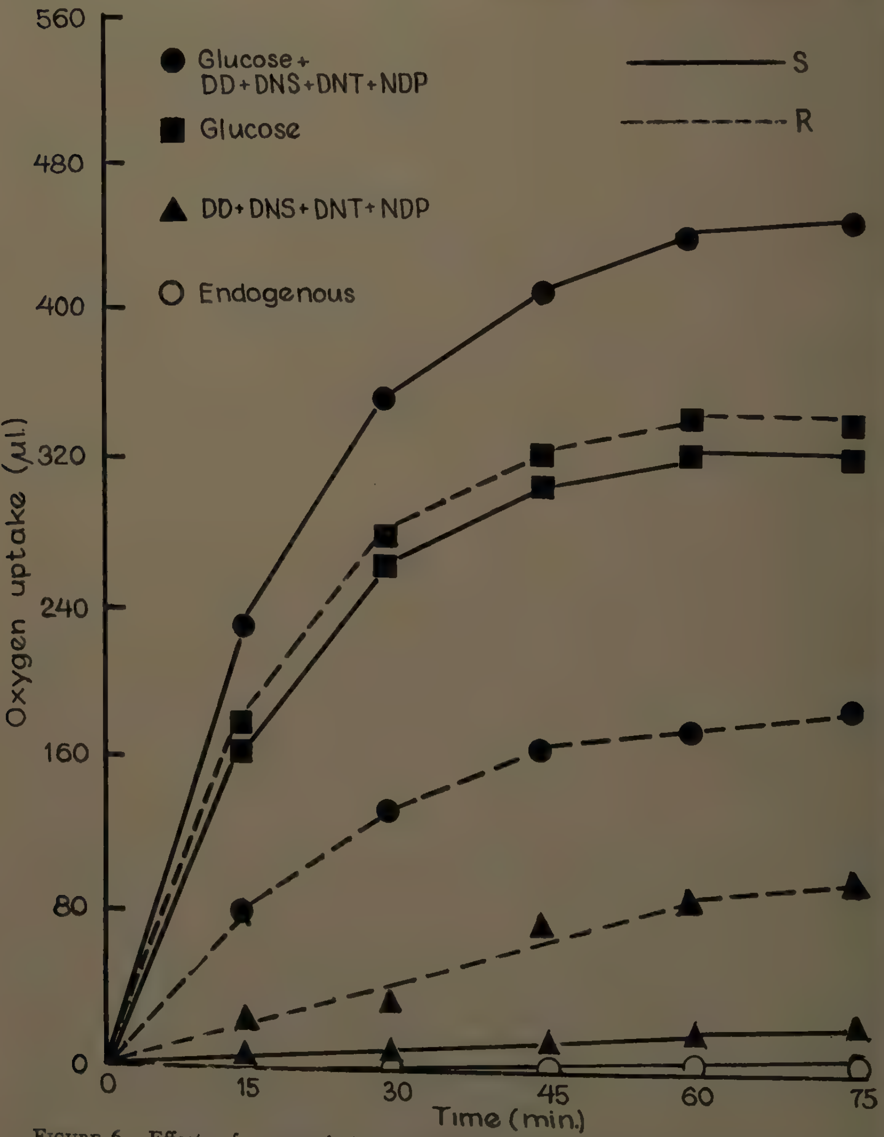

FIGURE 6. Effects of enzymatic DNA digests, deoxynucleosides, deoxynucleotides, and nucleoside-di-phosphates on glucose oxidation of type I virulent and avirulent pneumococci.

additional treatment. Further proof for the natural role of these factors could be obtained if it became possible to isolate active compounds from animals during the course of infection to utilize them for the stimulation of the growth of virulent cells *in vitro*.

The actual mode of action of the enhancing effects of the full supplementation system on DNA synthesis of virulent cells will require further elucidation. For example, the breakdown products may serve two important functions: (1) to provide the necessary energy for incorporation of extracellular precursors by stimulating the oxidation of glucose and (2) to provide the necessary precursors for DNA synthesis. Which portion of the full supplement stimulates glucose oxidation and which is responsible for enhancing DNA synthesis is not entirely clear. However the present results indicate that DD + DNT act as the stimulators of respiration, whereas DNA mixtures supply the precursors. NDP mixtures appear to have little effect in the resting cell system in contrast to their role in the growth system (Firshein and Braun, 1960). The factor(s) present in the DNA digest does not appear to be identical with any component found in the remainder of the full supplement, since the addition of the digest to such supplements enhances respiration and DNA synthesis to a greater degree than that exhibited by these latter components when they are employed by themselves. Furthermore, the results obtained with C^{14}-DNA digest preparations and those obtained with variations in the addition of the extracellular DNA supplement also indicate that the active material is controlling DNA synthesis in a manner other than as a supplier of precursor material. It is interesting to note in this respect that Bessman *et al.* (1958) observed in studies concerning the cell-free synthesis of DNA by enzymes extracted from *Escherichia coli* that a polymerized DNA primer required in the reaction could be replaced or activated severalfold by a DNase-treated DNA. Previous results (Firshein and Braun, 1960) have shown that the digest could be chromatographed by ion exchange into four separate fractions depending on molecular weight (Bendich *et al.*, 1955). The small molecular weight fraction was found to produce the enhancing effects on S cells provided Mn^{++} was present. Therefore, it would be of interest to isolate the active factor from a labeled DNA preparation and to ascertain its exact metabolic role.

It is interesting to speculate on the manner in which the observed net increase in the DNA per cell manifests itself morphologically in the virulent *Pneumococcus* cell. It is possible that it might involve the formation of more nuclei per cell. Certain genetic experiments capable of analyzing this possibility are feasible and are being undertaken. Regardless of the manner in which the increased DNA per cell is organized, the question regarding the genetic competence of such cells must be answered. Transformation studies to be reported subsequently have indicated that the excess DNA formed is genetically competent.

The differential response of virulent and avirulent pneumococci to the nucleic acid breakdown products can be interpreted in several ways. First, it is conceivable that a difference in permeability is responsible for the inefficiency exhibited by R cells. This may be due either to vastly dissimilar charges present on the cell surfaces of S and R cells or to the presence of specific permeases in the former cell type. If these hypotheses are correct, studies with cell-free preparations, or possibly protoplasts, should reveal no significant differences between S and R pneumococci in respiratory activity or DNA synthesis in the presence of the full supplementation system. On the other hand, if such studies still reveal a greater response in S cells, a logical assumption would be that either the enzyme systems are more efficient in virulent cells or that alternate pathways to DNA exist in avirulent cells that do not depend on the presence of the extracellular nucleic acid breakdown products employed in these experiments. The fact that such cells appeared to utilize glucose and casitone for metabolic purposes (including DNA synthesis) to a much greater degree than their virulent relatives indicates that precursor requirements for DNA synthesis may be dissimilar. Another plausible explanation for the differences between the two cell types is based on the differential response exhibited in oxygen uptake levels when the nucleic acid breakdown products are present in the absence of glucose. Avirulent cells may simply be incapable of utilizing the extracellular supplements for DNA synthesis because these compounds are oxidized first and hence are unavailable for polymerization. In contrast, virulent cells are capable of using the precursors for DNA synthesis because they are unable to oxidize them extensively in the presence or absence of glucose. In relation to these differences, it will become important to explain why the full supplementation system depresses oxygen consumption in avirulent cell suspensions containing glucose, whereas, as described above, the full supplementation system is oxidized to a greater degree in avirulent cell suspensions in the absence of the sugar. If the same oxidative enzymes are employed for both oxidations and both utilizable substrates are available (glucose and the breakdown products), it is possible that there may not be sufficient enzymatic raw material to react with both substrates at the same time. This interpretation would assume that: (1) the oxygen consumed in the oxidation of glucose is much greater than that produced by oxidation of the nucleic acid breakdown products and (2) both supplements, that is glucose and the breakdown products, if present together, must be oxidized simultaneously, thus lowering the over-all oxygen uptake values. The results have substantiated the first point (avirulent cells oxidize glucose to a much greater extent than they do the extracellular precursors), but it would be necessary to isolate the oxidative enzymes from avirulent pneumococci in order to determine whether the second point is valid (whether such enzymes must act on both substrates when they are present in combination).

The action of Mn^{++} on respiratory activity deserves additional comment, although its effects were mentioned only briefly in the results. It is surprising that this ion depresses oxygen uptake of avirulent cells in the presence of glucose, since it is well known that Mn^{++} is involved in general respiratory mechanisms (Ochoa and Weisz-Tabori, 1948). Thus it is possible that, although respiratory pathways are probably similar in both cell types, rate-limiting steps may be the cause of the deleterious effects of Mn^{++} in avirulent cells. It would be desirable to test the effects of Mn^{++} on the known reactions with which this ion is involved, using enzymes extracted from both virulent and avirulent pneumococci.

In conclusion, these results present problems that can be solved only by further elucidation of the enzyme systems involved in respiration and enhanced DNA synthesis. There are, undoubtedly, many other factors involved in elucidating biochemical differences between virulent and avirulent pneumococci that have not yet been recognized. However, despite such deficiencies in our knowledge, it can be hoped that investigations along the lines here described may assist in the early achievement of a better understanding of factors essential to an adequate evaluation of the metabolic basis of some aspects of virulence.

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Discussion of the Paper

R. AUSTRIAN (*Downstate Medical Center, State University of New York, New York, N. Y.*): Study of the capsular genome of pneumococcus has shown that it controls a multiplicity of biochemical reactions essential to the synthesis of capsular polysaccharide. Mutations affecting the synthesis of uronic acids are an important cause of loss of capsulation by strains of pneumococcus type I and type III. Exposure of noncapsulated mutants of pneumococcus type III to DNA from capsulated type I pneumococci may be followed by the appearance of transformed cells producing two types of capsular polysaccharide. Binary capsulation in pneumococcus provides a new basis for serologic cross-reactivity in this species. The phenomenon of binary capsulation results from the simultaneous presence within the pneumococcal cell of two capsular genomes, one mutated and the other normal. Interaction between the biochemical pathways controlled by the two capsular genomes leads to augmentation of the product controlled by one and to partial suppression of that determined by the other. An example of genetic augmentation and suppression is, therefore, explicable in biochemical terms.

BIOCHEMICAL PROPERTIES OF VIRULENT AND AVIRULENT STRAINS OF *MYCOBACTERIUM TUBERCULOSIS**

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Introduction

In seeking information on the mechanisms involved in microbial pathogenicity, comparative studies of virulent and avirulent mutants of the same parent strain are often used to good advantage. Any differences observed between such variants may be worthy of investigation as to their possible role in virulence. The method has, however, two serious inherent weaknesses:

First, work must be done with bacteria grown *in vitro* since avirulent variants would usually not multiply *in vivo*. Significant differences have been shown to exist between various kinds of pathogens grown *in vitro* and organisms of the same strain separated from the organs of infected animals. Since such differences exist, a note of caution must be entered. Most of our biochemical knowledge concerning pathogens was gained from studies on organisms grown *in vitro*. We are unable at the present to tell whether some conclusions may not have to be revised in the light of work with *in vivo* grown organisms.

Second, it is well known that the composition of artificial growth media may profoundly influence the chemical make-up and the biochemical activity of microorganisms. This circumstance must be taken into account because, in many instances, the significance of certain findings will be limited to the very conditions under which the observations were made.

With these two serious reservations in mind, consideration will now be given to the differential biochemical properties of virulent and avirulent tubercle bacilli.

Virulence in the present discussion is defined as the ability of microorganisms to multiply in a host and to cause a progressive disease. Avirulent organisms not only fail to multiply but are rapidly eliminated from the tissues of the host. Microorganisms showing an intermediate behavior are called attenuated. They might multiply to a limited extent and cause minimal lesions, but they will be eliminated in the end and do not cause progressive disease. By definition, then, an organism may be virulent for one host species and attenuated or avirulent for another.

To stress this point is not merely academic sophistry, but is important in the framework of the present discussion. What is meant by virulent and avirulent strains must be clearly understood. Especially in connection with modern chemotherapy, the question whether isoniazid-resistant tubercle bacilli are virulent has great practical interest. We shall return to this particular case later.

The subject of the virulence of tubercle bacilli has been previously reviewed.[1,2] In accordance with the subject of this monograph, the present

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discussion is concerned only with the biochemistry of virulent and avirulent tubercle bacilli.

Substrate Response and Enzymatic Activity

In general, pathogens are more fastidious and selective in their growth requirements than saprophytes. In the course of their phylogeny, parasites may lose some synthetic functions,[3] thus becoming more specialized in their growth requirements. For mycobacteria, Hanks and Gray have shown that there is a pattern of diminishing oxidative response to various substrates.[4] TABLE 1, which is from their article, illustrates this fact. It is seen from this table that there is a pattern of diminishing substrate response from saprophytes over attenuated strains to parasites, but it would obviously make little sense to conclude from these findings that human leprosy bacilli are more pathogenic than murine leprosy bacilli or Johne's bacillus. The three kinds of organisms are pathogenic for three different animal species, causing three distinct and characteristic disease entities. However, within a single bacterial species, a similar comparison might become meaningful. A few examples of comparable data on metabolic activities of various strains of tubercle bacilli differing in their virulence have been compiled in TABLE 2. In all of these instances, there was an inverse proportionality between the relative virulence of strains of tubercle bacilli and their readiness to react to a given substrate. The more virulent a strain, the more sluggish its response. Wherever saprophytic mycobacteria were included in the investigation, they were in turn considerably more active than even the avirulent tubercle bacilli. It seems that virulent strains not only have a lower respiratory rate than avirulent organisms, but that, in addition, their respiration is less inhibited at lower oxygen tensions than that of avirulent organisms.[9a] The same is true for growth rates *in vitro*[9b] and growth and survival rates *in vivo*.[9c] Low oxygen tensions affect the multiplication of virulent strains to a lesser degree than that of attenuated or avirulent organisms. It may well be that the dependency of avirulent strains on relatively high oxygen pressures is a factor in their inability to survive and multiply in tissues where virulent organisms are able to proliferate.

TABLE 1[*]

THE PATTERN OF DIMINISHING OXIDATIVE RESPONSE AMONG THE PATHOGENIC MYCOBACTERIA

Substrates enhancing O_2 utilization	Saprophytes	Intermediates	Tubercle bacilli	Johne's bacillus	Leprosy murine	Leprosy human
Succinate	++++[†]	–	–	–	–	?
Acetate	++++	+++	±	–	–	?
Long chain fatty acids	++++	+++	++	–	–	?
Intermed. chain fatty acids[‡]	++++	+++	++	+	–	?

[*] Reproduced by permission of *Advances in Tuberculosis Research*.[4]

[†] The degree of oxygen consumption is indicated in an arbitrary scale ranging from – to ++++.

[‡] Octanoic.

Differences in metabolic activity are also expressed in the speed of bacterial multiplication. Saprophytic strains grow much faster than tubercle bacilli. On the other hand, it is questionable whether there are significant differences in the generation times of virulent and avirulent tubercle bacilli, and the analogy cannot be pursued further.

In comparing organisms of the $H_{37}Rv$ strain grown *in vitro* with bacteria of the same strain separated from the lungs of tuberculous mice, Segal and Bloch[10] found that the *in vivo* grown organisms were more virulent for mice than comparable suspensions of bacteria grown *in vitro*. It might therefore be permissible to consider *in vivo* and *in vitro* grown bacteria as substrains of different virulence and to compare their biochemical activities in the framework of the present discussion. Generally, the differences in metabolic activity, as measured by the oxygen uptake of the bacteria, were similar to those found between other pairs of bacterial strains differing in their virulence: the more virulent *in vivo* grown organisms were again more sluggish and more selective in their substrate response than were the less virulent test tube-grown organisms.[11]

TABLE 2

SOME METABOLIC ACTIVITIES IN STRAINS OF *M. TUBERCULOSIS* OF DIFFERENT VIRULENCE

Activity measured	Relative activity* in				Reference
	Virulent TB	Attenuated TB	Avirulent TB	Saprophytic mycobacteria	
Reduction of 2,3,5-triphenyltetrazolium	−	+	++	+++	5,7
Reduction of methylene blue	+	++	+++	++++	6,7
Reduction of dichlorophenol-indophenol	−		++		8
Stimulation of O_2 consumption by lactate	+	++	+++	++++	9

* The relative activity is expressed in an arbitrary scale ranging from − to ++++.

FIGURE 1 illustrates these findings. The endogenous respiration was the same for the bacteria grown *in vivo* and *in vitro*. They all had a QO_2 of about 5.0. However, as is evident from FIGURE 1, the substrate response of the two kinds of suspensions was quite different. A number of substrates to which the *in vitro* grown organisms responded readily by increased oxygen consumption did not affect the respiration rate of the *in vivo* organisms at all. In this respect, they resembled more the murine leprosy bacilli[4] than any known strain of tubercle bacilli. There were two notable exceptions in the limited number of substrates that Segal and I investigated: sodium salicylate and oleic acid stimulated the oxygen uptake of *in vivo* organisms in the same degree as those grown *in vitro*. The same was true for two other fatty acids, n-heptanoic and octanoic acid, whereas benzoates other than salicylate, which did stimulate the *in vitro* organisms, failed to influence the oxygen uptake of *in vivo* grown bacteria.[11]

In our experiments, we were working with whole bacteria rather than with cell-free extracts. This makes the evaluation of these results more difficult since permeability factors might conceivably be responsible for some of our results. Especially in the case of tubercle bacilli, cell wall permeability has always been thought to be a particularly critical factor.

There was no indication in our experiments that the lack of substrate response was due to the effect of an inhibitor. Adding various lung extracts to *in vitro* grown bacteria did not influence the oxygen uptake of these. Moreover, the fact that the *in vivo* bacteria did respond to fatty acids and salicylate indicated that, for some substrates at least, their respiratory mechanism was intact, but that they were more specific in their choice than the test tube-grown bacteria.

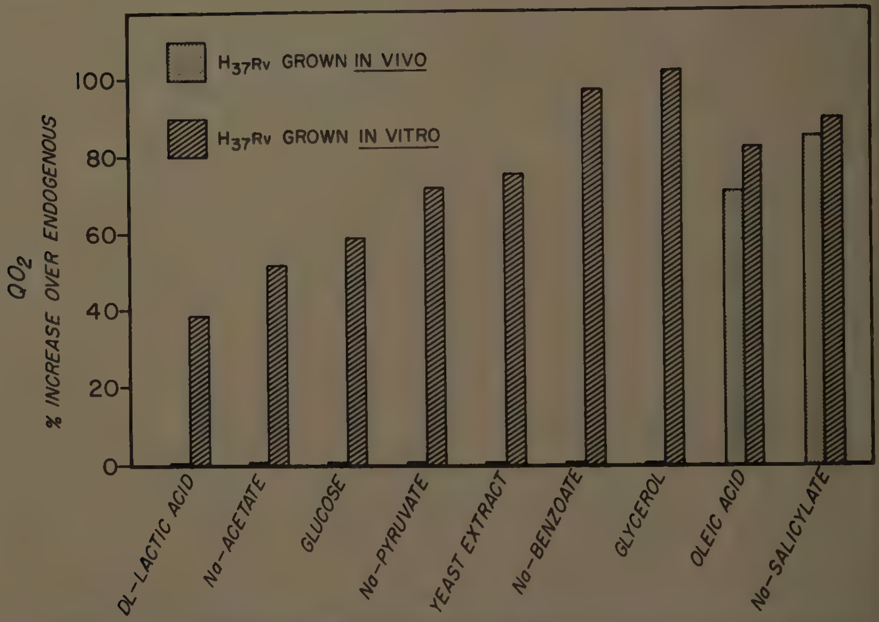

FIGURE 1. The respiratory response to various substrates of $H_{37}Rv$ grown *in vivo* and *in vitro*. The columns indicate per cent increase of QO_2 over endogenous.

Recently two Israeli workers, Bekierkunst and Artman,[12] used our technique for the separation of tubercle bacilli from the lungs of infected mice. Instead of working with whole bacterial cells, they prepared cell-free extracts and measured the hydrogen transfer capacity of these in the presence of various substrates, using the reduction of triphenyltetrazolium as an indicator. Just as had been found with whole cells, the cell-free extracts from *in vivo* organisms showed no dehydrogenase activity in the presence of lactate, malate, and pyruvate, whereas extracts from normally grown tubercle bacilli or BCG were active. These investigators suspected that an inhibitor present in these extracts might be responsible for the lacking substrate response. Indeed, they found in the cell-free extracts from organisms separated from infected mouse lungs an activity that inhibited the lactic acid dehydrogenase of other mycobacteria and also the succinooxidase activity of normal mouse lung homogenates. Analogous extracts from *in vitro* grown tubercle bacilli or BCG, on the other hand, did not inhibit their enzyme activity. It is reasonable to assume that the inhibitor that interfered with the lactic acid dehydrogenase from BCG was identical with the inhibitor of the succinooxidase from lung tissue. In a recent letter Bekierkunst informed me that an inhibitor of dehydrogenase activity was also found in supernates from homogenates of infected lungs. In our earlier study[5] we had checked whether the separation procedure itself might be responsible for the different results obtained with *in vivo* and *in vitro* grown organisms. In the course of these control experiments no inhibiting activity was found in homogenates of mildly infected lungs that would affect the oxygen uptake of *in vitro* grown bacteria. However, in the light of the recent findings of Bekierkunst and Artman, this question will have to be reconsidered. Especially, the problem needs to be clarified as to whether the inhibitory activity, that is present in and affects the activity of cell-free extracts, can also be shown to affect the oxygen uptake of whole bacterial cells or whether the limited substrate response of *in vivo* grown tubercle bacilli is due to the presence of an inhibitor in the tissues of heavily infected lungs.

All enzyme studies become more significant if carried out with cell-free, purified systems rather than with whole cells. A great deal of work has been carried out with cell-free extracts from mycobacteria, but practically all of it was done with extracts from saprophytic or avirulent mycobacteria. Goldman[9] has recently completed a comprehensive review of all enzyme systems that have been studied in mycobacteria. Unfortunately, most of these studies are unrevealing in the framework of the present discussion. Comparisons were seldom made between enzyme systems from virulent and avirulent organisms, and thus these studies fail to shed light on biochemical characteristics that might differentiate such strains.

Chemical Composition

Differences in the chemical make-up of various mycobacterial strains have long been known to exist. Such differences may be reflected in quantitative variations in the total yield of certain classes of bacterial constituents, such as lipids, proteins, and polysaccharides. They may express themselves in finer chemical distinctions that can only be detected by serological methods, or they may be shown by the presence or absence in the bacteria of single chemically defined components.

None of the first two criteria has revealed differences between various strains of tubercle bacilli that were consistently correlated with their degree of virulence.[13-15] The gross chemical composition of tubercle bacilli depends to some extent on the medium in which they were grown and also on the physiological state of the organisms at the time of harvest. Quantitative variations in the yield of specific groups of constituents that might exist between different strains are often largely overshadowed by variations between cultures of the same strain differing in age or in the method by which they were cultivated.

When taking the immune response of animals to injections of bacterial homogenates or culture filtrates as an indicator of chemical differences between strains, the results have been equally disappointing in regard to the present problem. While immunologic differences permit the grouping of tubercle bacilli according to the presence or absence of certain antigens, the resulting groups do not class the bacteria by their virulence.[15] A truly enormous amount of work has gone into analyzing the antigenic structure of tubercle bacilli, but not one antigen has been found that appears to occur in virulent organisms alone. It is almost tempting to conclude that any cellular component characteristic of virulent strains alone would either be nonantigenic or would not be produced under the commonly used laboratory conditions of culturing bacteria.

Nonantigenic bacterial constituents have, however, been found that occur preferentially or exclusively in virulent organisms. Several years ago, I described a lipid obtained by surface extraction of virulent cord-forming tubercle bacilli with petroleum ether that I termed cord factor.[15] The ability of tubercle bacilli to grow in a peculiar cord or serpentinelike arrangement had long been known, but it was Middlebrook *et al.*[16] who first pointed out that this morphologic pattern was typical of virulent strains. The lipid material that was extracted from these organisms proved to be highly toxic for mice. An effort was made to identify the toxic factor chemically. In collaboration with Noll *et al.*[17] the constitution of cord factor was completely cleared up. It was recognized as trehalose-6,6′-dimycolate and the structure was confirmed by synthesis (Gendre *et al.*[18]). The toxicity of other lipid fractions of tubercle bacilli could be traced to contamination with cord factor.[19] These studies emphasize the importance of attempting to identify interesting biological compounds chemically wherever this is feasible. This is especially true in this particular field of bacteriology where a great deal of confusion and controversy could be avoided if the help of competent chemists were to be sought more often.

The studies of Lederer and his group[20] and of Noll[21] have also shown that certain structural variants of trehalose-6,6′-dimycolate have a biological activity similar to that of the original material. This is not particularly surprising. Similar biological activities of chemically related compounds are frequently observed. This fact merely indicates that our biological assay methods are not sufficiently sensitive to distinguish between individual members of a family of high molecular constituents.

The toxicity of cord factor is high but of a delayed type; 5 to 10 μg. kill adult mice. The animals die 5 to 8 days after injection. Repeated small doses are more toxic than a single large one. If the dosage is increased above the LD_{50}, toxicity does not increase accordingly. The cause of death is unknown. Extensive pulmonary hemorrhages are the most conspicuous symptom. It is not easy to assess the significance of the toxicity of cord factor. Kato and his coworkers[22,23] have recently found that cord factor affected the activity of a number of enzymes. After intraperitoneal injection into mice of 5 μg. of cord factor, the activity of succinic dehydrogenase in liver homogenates was significantly decreased over a period of several days. Malic, lactic and α-glycerophosphoric dehydrogenase activity was likewise inhibited in liver homogenates as well as in mitochondrial preparations from liver. As with the direct toxicity, the effect was not enhanced by higher doses. In order to inhibit these enzymatic activities, cord factor had to be injected. Adding it *in vitro* to liver homogenates was without effect.

This observation seems important in the light of earlier experiments of Martin and his group.[24,25] These authors found a reduced succinic dehydrogenase activity in the kidneys of guinea pigs infected with virulent tubercle bacilli. With progressing tuberculosis, the enzyme levels became gradually lower until the animals died. The decrease in enzyme activity paralleled the severity of the experimental disease. Infection with BCG caused a temporary decrease in enzyme level from which the animal recovered within a few weeks. Both Martin as well as Kato and their colleagues isolated soluble tissue factors from normal liver and kidney that restored the enzyme activity reduced under the influence of cord factor or infection. It is conceivable that the two tissue factors are identical and that the tuberculous injury affects this particular biochemical activity. Kato *et al.*[26] have added strong support to this view by recent work showing that the succinic dehydrogenase activity of mouse liver is decreased during infection with virulent bacilli but not with BCG, just as they had earlier found that it was decreased after injection of cord factor. Likewise, the injection of heat-killed tubercle bacilli into mice resulted in a decrease of succinic dehydrogenase whereas the injection of heat-killed attenuated or avirulent tubercle bacilli or saprophytic mycobacteria had no such effect. Finally, virulent organisms from which cord factor had been removed by surface extraction with petroleum ether lost their inhibiting activity for succinic dehydrogenase, but gained it again after they were recoated with cord factor. Some of these findings are illustrated in FIGURES 2 and 3.

Also remembered in this connection are the findings of Bekierkunst and Artman[12] who isolated a potent inhibitor of succinooxidase from tubercle bacilli separated from infected mouse lungs. The results that were obtained independently by these three groups of investigators suggest that the inhibition of dehydrogenase systems may indeed be an important metabolic lesion characteristic of tuberculosis and that this biochemical injury is mediated by cord factor.

Further indirect evidence for the role in pathogenicity of the chloroform soluble waxes of which cord factor is a constituent was brought about by elimination studies of P^{32}-labeled virulent and avirulent tubercle bacilli. Yamamura *et al.*[27] found that the injection of killed, labeled virulent bacteria resulted in a much slower elimination of P^{32} than the injection of killed, labeled avirulent organisms. Removal from the bacteria of the chloroform-soluble waxes that contain the cord factor eliminated the differences between virulent and avirulent organisms as measured by the excretion rate of $P.^{32}$

It seems, therefore, that there is an increasing body of evidence indicating that cord factor plays a role in the mechanisms of virulence of tubercle bacilli. It is perhaps good to re-emphasize that this is not to be regarded as the only mechanism of virulence. It is one of probably many, the mode of action of which is just beginning to be understood.

Recently, Middlebrook *et al.*[28] described a sulfolipid material that they isolated from virulent tubercle bacteria and that was not found in avirulent strains. The material that is nonantigenic has a sulfonic acid group. Its biological significance cannot be evaluated at the present time.

Isoniazid Resistance and Virulence

Soon after the introduction of isoniazid into the therapy of tuberculosis, it became apparent that drug-resistant strains of tubercle bacilli emerged frequently in the course of treatment. Isoniazid resistance develops in a single step. The mutation rate is of the order of 1 to 3 × 10^{-6} per bacterium per generation, a value that is 1000 times higher than for streptomycin for example.[29] The frequency with which such strains appeared and the fact that many of them were double and triple mutants, resistant not only to isoniazid but also to streptomycin, *p*-amino-salicylic acid, and other drugs, soon provided an entirely new class of heretofore unknown variants of tubercle bacilli that were selected under the pressure of the potent new drugs. These mutants differed in many important aspects from the parent wild strains. The fact that many of them were less virulent for experimental animals than their parent strains justifies their discussion in the present context.

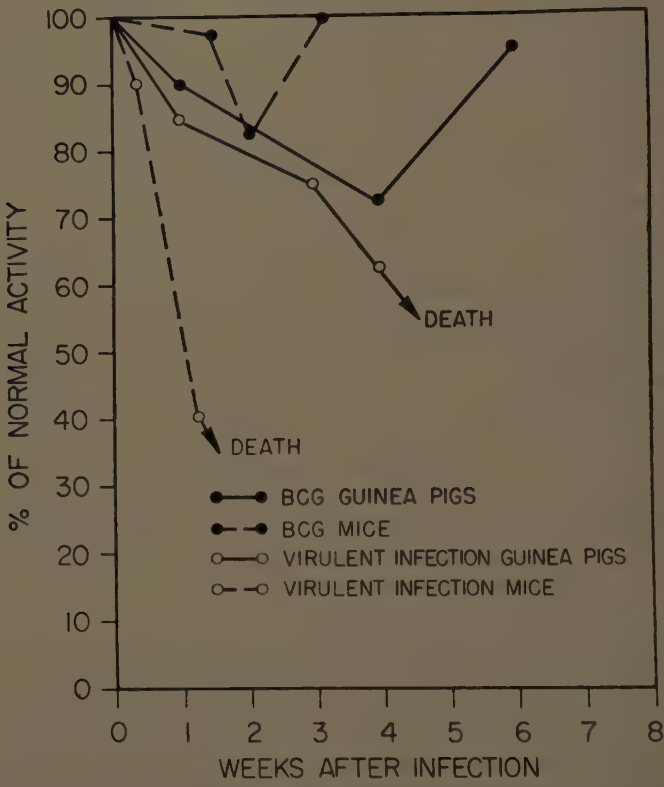

FIGURE 2. The succinic dehydrogenase activity in the liver of mice and guinea pigs after infection with virulent tubercle bacilli. The figure is compiled from the data of Kato *et al.*[26] and Chaudhuri and Martin.[24] Reproduced by permission of the *American Review of Respiratory Diseases* and the *Journal of Experimental Medicine.*

Most isoniazid-resistant strains have lost their virulence for guinea pigs.

This fact was first observed by Middlebrook and Cohn[30] and has since been confirmed many times.[31] However, strains that are avirulent for guinea pigs might still be virulent for mice and other species. This was first reported by Bloch *et al.*[32] and has also often been confirmed.[31] For obvious reasons, the virulence of isoniazid-resistant tubercle bacilli for man is difficult to ascertain, but recent studies in infants show beyond doubt that primary tuberculosis can be caused by isoniazid-resistant tubercle bacilli and that at least some of these resistant mutants are fully virulent for man.[33] As time goes on and epidemiologic conditions favor the spread of these organisms, we must undoubtedly expect to see more and more primary infections caused by such strains. Thus it is not permissible to equate isoniazid resistance with lack of virulence. While the two characteristics often go together, they do not seem to be genetically linked.

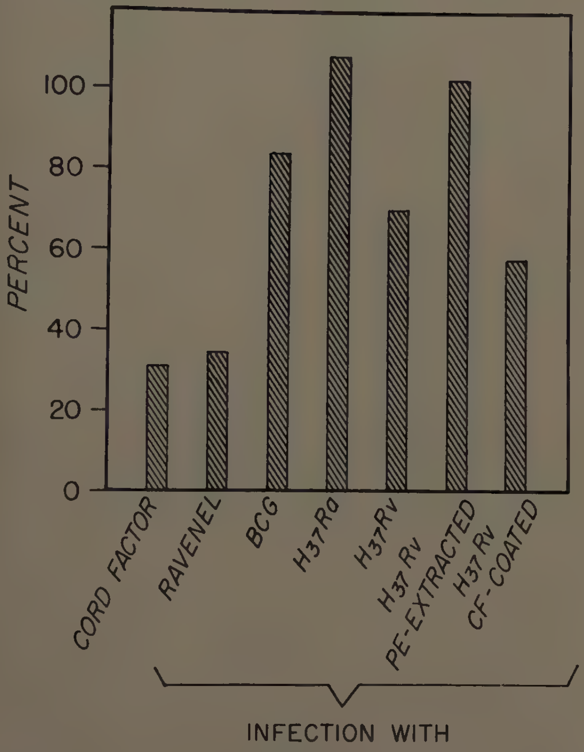

FIGURE 3. The succinic dehydrogenase activity in mouse liver after injection of 0.01 mg. cord factor or infection with virulent (strain Ravenel), attenuated (BGG), and avirulent (strain $H_{37}Ra$) tubercle bacilli, and with virulent (strain $H_{37}Rv$) bacteria before and after extraction with petroleum ether (PE) and after coating of the extracted bacteria with cord factor (CF). Data from Kato *et al.*[23,26]

Nevertheless, there are resistant mutants that have lost all virulence for experimental animals and that are characterized by other biochemical properties distinguishing them further from virulent wild strains. One of the most constant features of isoniazid-resistant tubercle bacilli is their reduced catalase activity.[34] Although exceptions have been noted, there is generally a very good agreement between low catalase activity, low virulence for guinea pigs, and high isoniazid resistance of tubercle bacilli. Similarly, the activity of the closely related enzyme peroxidase parallels the isoniazid resistance of tubercle bacilli.[35] In isoniazid-susceptible strains, positive peroxidase tests correlated more closely than positive catalase tests, whereas in isoniazid-resistant strains the incidence of negative catalase and negative peroxidase tests was the same.[36] It is conceivable that the lack of catalase and peroxidase in isoniazid-resistant bacteria makes these organisms more susceptible to hydrogen peroxide they may encounter in host tissues and that this may be a reason for their reduced virulence. Enhanced susceptibility of isoniazid-resistant tubercle bacilli to the lethal effect of hydrogen peroxide has indeed been demonstrated.[37] On the other hand, it is not infrequent for tuberculous patients to excrete catalase-negative organisms for months or years. In these cases, the lack of catalase does not seem to interfere with the ability of the bacteria to multiply *in vivo*. Moreover, other avirulent mutants of tubercle bacilli, which are isoniazid-sensitive, possess intact catalase and peroxidase systems. There must obviously be many mechanisms by which virulence or lack of virulence asserts itself.

Some isoniazid-resistant strains are more fastidious in their growth requirements than the sensitive parent strains. Thus Fisher[38] found that some resistant variants needed a growth factor that was present in bovine albumin, Fraction V, and that he was later able to identify as hemin.[39] Other growth requirements characterizing isoniazid-resistant mutants were described by Cohn *et al.*[40] It should be emphasized that none of these requirements is a constant feature of every isoniazid-resistant mutant. Such mutants are often found to be associated with resistance but, like virulence, they seem to be genetically independent. The powerful means of selecting mutants by isoniazid has provided a host of strains that, by the very way by which they were selected, are all resistant to this drug and that, in addition, often share other properties, such as the absence of catalase and peroxidase activity or the lack of virulence for guinea pigs. The frequency with which these properties are associated suggests closeness of the loci where the respective genes are located. This situation is strikingly different from the case of streptomycin-resistant mutants, where there are no comparably frequent variations in enzyme mechanisms or modifications of virulence associated with drug resistance.

Atypical Tubercle Bacilli

As a consequence of antituberculosis chemotherapy, the bacteriologists have recently been confronted with a whole new class of mycobacteria, the so-called atypical, chromogenic tubercle bacilli. These organisms appear with increased frequency in pathological materials from patients. Doubtless they are seen more often because the selection pressure of antituberculous drugs favors their emergence from a much greater population of drug-sensitive tubercle bacilli. By necessity then, these chromogens are drug-resistant. They vary in their virulence for experimental animals. Mostly, they are nonpathogenic. Again, the question of their virulence for man is more difficult to answer. There is no doubt that, in some clinical cases, chromogens must be regarded as the agents causing the disease, whereas recent epidemiologic studies[41] suggest that inapparent infections with such mycobacteria seem to be very widespread in certain geographic areas. These bacteria would therefore qualify as further objects for study in the present context, but they can be mentioned here only as prospective models for gaining more information on the nature of mycobacterial virulence since, at the present time, too little is known about these organisms. Their classification, especially, is still in a very preliminary state, and we do not really know how closely related these bacteria are to *M. tuberculosis*, the subject of this presentation.

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THE BIOCHEMICAL PROPERTIES OF NONCULTIVATED MYCOBACTERIA

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A discussion of noncultivated mycobacteria is retarded by lack of the kind of data that results from study of the more independent microbes, including the familiar species of mycobacteria. Perhaps a small contribution can be made by trying to bring into perspective the factors that seem to force pathogenic mycobacteria toward host dependence and noncultivable states and by outlining a few of the properties of such organisms.

Noncultivable mycobacteria have been reported in Bolivian bull frogs (Machicao, 1954), Mexican green parrots (Wherry, 1920), Scandinavian wood cocks (Christiansen, 1946; McDiarmid, 1948), Dutch East Indian water buffalo (Lobel, 1938), cutaneous tuberculosis and certain resected lung lesions (Hobby *et al.*, 1954), Johne's disease of sheep and cows (A. B. Larsen, personal communication) and, most notoriously, rat and human leprosy.

The devices whereby tubercle bacilli maintain capacity for independent proliferation (and escape the dilemma of host dependence) may be supposed to rest upon properties that permit more frequent escape from intracellular environment. The most important of these seem to be toxic lipids (Block, 1950; Middlebrook *et al.*, 1959; Spitznagel and Dubos, 1955) that impair oxygen consumption by host cells (Martin *et al.*, 1955) and incite high levels of cellular sensitivity as a further means of existing on ulcerated surfaces. Although tubercle bacilli survive and grow within cells, the eugonic types are regarded as species that secure intermittent access to atmospheric oxygen.

The more host-dependent species, on the contrary, occur almost exclusively in nonpulmonary tissue. They may be regarded as more rigorously selected single or multiple step mutants that subsist, perhaps continuously, without normal access to oxygen and that may not employ oxygen as their major or sole acceptor of hydrogen and electrons.

Johne's bacillus, which excites extensive lesions in the ileum and lymph nodes (rather than the lungs) of sheep and cattle, represents the transitional stage in the evolution of mycobacteria towards strict host-dependence. Twort and Ingram in 1912 showed that *in vitro* propagation of this species requires a vitaminlike lipid complex that can be derived from cultivable mycobacteria provided they are grown under carefully specified conditions. The report by Woolley and McCarter (1940) that growth is facilitated by naphthoquinones has not been confirmed. Francis *et al.* (1953) and subsequent workers, have shown the essentiality of complex (870 mol. wt.) lipid extractives containing hydroxamic acid. This complex is extractable in acetone or hot methanol. It has been crystallized in the presence of aluminum ions and is designated as mycobactin. Its most interesting properties are: (1) its capacity to chelate metals in fixed molar ratios (one atom per molecule of mycobactin), and (2) its solubility in lipids and lipid solvents. The way in which this complex trans-forms Johne's bacillus from a noncultivable to a cultivable state remains to be elucidated.

Studies on the respiration and hydrogen transfer capacity of *Mycobacterium lepraemurium* (the causative agent of rat leprosy) have brought the ineptitudes of such species into sharper focus. The work of Gray and Hanks (1954) revealed the following properties: (1) failure to enhance metabolic activity or to achieve maintenance in the presence of the usual classes of biologically useful sources of N or C (Gray, 1952; Hanks, 1954*a*); (2) facilitation of endogenous metabolism by yeast and liver extracts (Gray, 1952); (3) relief of endogenous inhibitions and improved stabilization in the presence of purified serum albumin (Gray, 1952; Hanks, 1954*a*); (4) preservation of infectiousness by refrigeration in serum albumin and yeast supplement (Hanks, 1954*b*); and (5) prompt impedence of endogenous metabolism in the presence of serum from any of six animal species (Hanks and Gray, 1954).

Ito and Sonoda (1957*a*) have confirmed the failures of substrate utilization, the stimulation by yeast, liver and other tissue extracts, and the marked toxicity of serum. They also have demonstrated (1958) the presence of catalase, the liberation of NH_4 from asparagine (1957*b*), and transaminations between asparagine and α-ketoglutarate.

The stimulating effect of tissue extracts on endogenous hydrogen transfer have been attributed by Kusaka (1958*a*) to heat-stable components derived from the mitochondrial fraction of liver cell homogenates. In the presence of these crude cofactors slight enhancements of dehydrogenation or oxygen consumption were observed upon adding glucose, succinate, palmitate, glutamate, or glycine. In attempts to explain the action of mitochondrial extracts, Kusaka (1958*b*) found the pyridine coenzymes DPN and TPN stimulated primarily the endogenous dehydrogenations. Flavine adenine dinucleotide was implicated in the dehydrogenation of succinate because of partial inactivation of mitochondrial extracts by exposure to ultraviolet light. Cell-free extracts of *M. lepraemurium* were found to contain also dehydrogenase for glucose-6-phosphate, which had been inactive with whole cells. This activity was demonstrable only in the presence of heated mitochondrial extracts.

Tanemasa and Tsutsumi (1958) investigated the capacity of *M. lepraemurium* to enhance dehydrogenations in the presence of 181 natural plus nonbiologic compounds. Ortho-aminophenol, higher fatty acids (lauric, tridecanoic, and chaulmoogric), and indole and skatole were the only compounds that increased the yields of formazan.

Cytologic Studies

Definition of an anatomical property that impedes metabolic study, and may complicate cultivability, is perhaps useful. Investigations designed to assay the viability and metabolic activity of rat and human leprosy bacilli by observing microscopic deposits of formazan required that 100 per cent of the organisms, both colored and white, be clearly delineated. In all conditions to be described, routine application of surface films of Congo red or nigrosin has permitted distinction between the normal width of stainable rods and the total space occupied. For this reason it can be stated that impermeability is dependent upon the character distribution and amount of extrabacillary matrix or true capsules of the different species studied.

Working conditions have been as follows: drying of microdroplets for only 3 sec., prompt rehydration in tetrazolium blue acceptor systems or in dyes in physiological solutions, continuous incubation at 37° C., and determination of the percentages of bacilli that are reactive or are stained in unit time.

Impermeabilities to tetrazoles. In the case of the saprophytic *M. phlei*, responses to substrate, nutriment, or damage were readily assayed since the proportion of bacilli producing formazan were related directly to viability or expected response. Under optimal circumstance, such activity could be shown in 60 per cent of cells of BCG and 33 per cent of H-37Rv. In highly infectious suspensions of *M. lepraemurium*, however, only 3 to 9 per cent of the microorganisms could be made to exhibit activity. These results were explained by the failure of tetrazoles to penetrate into internal sites of metabolic activity. Fortunately 0.2 per cent of safranin-O could be incorporated in acceptor systems without modifying metabolic turnover. Combinations of tetrazolium blue and safranin revealed in *M. lepraemurium* suspensions three classes of bacilli: (1) small, penetrated rapidly and diffusely by safranin, and devoid of metabolic activity (inactive); (2) normal width, granular stains with safranin and one or two formazan granules (permeable but not in optimal condition); (3) twice the normal width, glistening white. Absolute numbers of which decreased during exposure to circumstances known to impair infectiousness. Exhaustive trials failed to disclose conditions that relieve the impermeabilities without destroying metabolic activity.

The superior quality of the impermeable cells was, however, demonstrated by heating such films in the presence of dyes. The previously impermeable class of bacilli now exhibited perfect rows of stained granules in numbers proportional to the length of the individual rods.

Impermeabilities to dyes. Incubation of *M. phlei* and *M. lepraemurium* in safranin-O (0.4 per cent) emphasized the marked differences between saprophytes and this pathogen. This dye stained 100 per cent of *M. phlei* cells within 1 hour, only 1 to 9 per cent of *M. lepraemurium* cells in 24 hours, and only 25 per cent during 10 days of continuous incubation.

Progressive staining of pathogens was secured with tri-phenyl methane dyes: basic fuchsins and crystal violet. Since the fuchsins did not yield stable aqueous solutions at physiological *p*H, crystal violet was used for broader comparisons of permeability.

Typical results are illustrated in FIGURE 1. Since 100 per cent of *M. phlei* cells stain within 27 min., broken lines are used in other curves to suggest the proportions of cells that may have stained within 30 min. Such cells, in respect to permeability, are analogous to saprophytes. Those that stained more slowly may be regarded as possessing the attributes of pathogens.

Several characteristics may be noted: (1) *M. phlei* presents but a temporary barrier to dye penetration and then stains at accelerated rates; (2) the impermeabilities of individual cells of pathogenic species differ so greatly that logarithmic times scales are required to plot results in a single chart. Orderly rates of penetration are seen only in *M. lepraemurium*, in which each bacillus is coated with an individual micro-capsule (Chapmans *et al.*, 1959), and clumping is rare. The sharper flexures in curves for BCG and *M. leprae* are in part induced. The clumping species share capsular components between cells and, when suspensions are prepared by emulsifying cultures or by scraping tissues, there is inevitably a disruption of natural relationships and the exposure of decapsulated bacilli.

The role of capsules in determining staining rates is readily observed in surface-coated preparations. *M. phlei* produces only enough capsular matrix to promote clumping; capsular matrices have not been seen. Thin or partial halos are seen only on the wider cells of BCG, as they begin to stain lightly between the 5th and 25th hours. Meanwhile the clumped bacilli usually are encased in a matrix that obscures visualization of individual cells. Partially stained *M. leprae* from nontreated patients may exhibit capsules as wide as the rods and appear 3 times as wide as the bacilli that stain rapidly. The microcapsules on *M. lepraemurium*, when expanded by heating at 50° F. for 30 min. in serum 0.5 per cent, become clear halos around all rods in which staining is being initiated. In any pathogenic species, during incubation in dyes, it is only as capsules are lost from individual rods that progressive staining occurs.

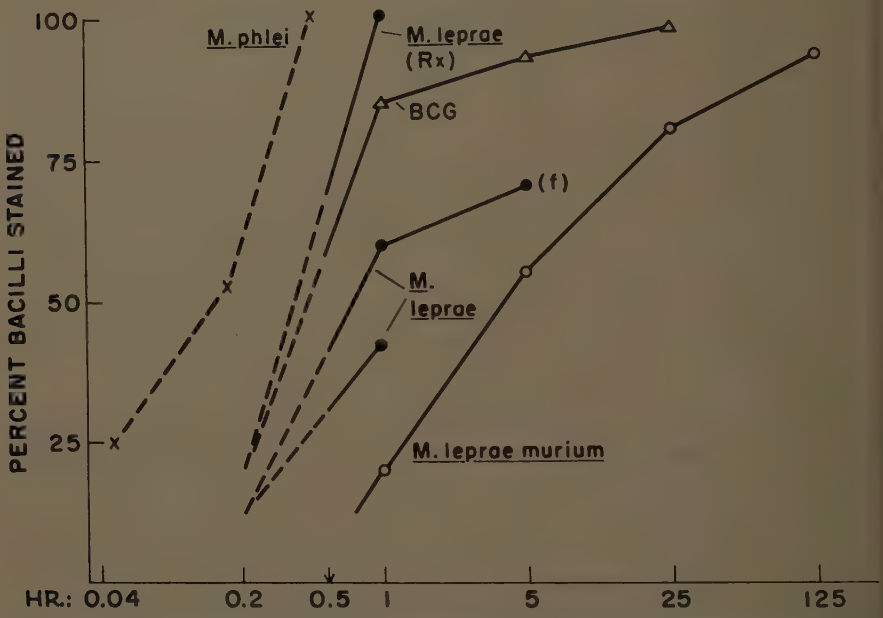

FIGURE 1. Relative impermeabilities of 4 species of mycobacteria. Staining rates in 0.04 per cent aqueous crystal violet, pH 7, 37° F. *M. leprae:* lower curve, direct from nontreated patients; middle curve, same but CO_2 frozen 6 months; upper curve, from sulfone-treated patients.

Efforts to remove capsules while conserving metabolic activity have served only to emphasize the inertness and ruggedness of these surface structures. The impermeability of *M. lepraemurium* cells has not been modified by any reasonable exposures to drying, acids and alkalies, Tween 80 or saponin, trypsin, pancreatin, ribonuclease or lysozyme, 24-hour immersions in petroleum ether, carbon tetrachloride or 1:1 ethyl ether in absolute alcohol, or by 7-day exposures to petroleum ether or carbon tetrachloride.

Three factors that cause rapid modification or removal of capsules from pathogenic mycobacteria are heat, phenol, and brief shaking of aqueous suspensions with only 5 to 10 per cent of chloroform by volume. Almost instantaneous declumping in such dilute chloroform indicates that waxes are a major constituent of capsules and that these components are readily released from the surface without exhaustive extraction by solvents.

Physiological implications. It is difficult to imagine the operation of energy-dependent ion transport beyond the limits of the plasma membrane in bacteria. In the case of mycobacteria, it may be suspected that components interfering with permeation occur in cell walls as well as on surfaces. The foregoing assays of permeation by strongly charged, highly colored cations are too artificial to duplicate the physiological problem of transporting highly charged but metabolically essential ions to the surface of plasma membranes in such cells. Nevertheless, these studies suggest that problems of this type might be critical in the two species that have been shown to possess the thickest (on *M. leprae*) and most uniformly impenetrable (on *M. lepraemurium*) capsular coatings.

General Comment

A survey of the biochemical properties of the host-dependent mycobacteria suggests that dramatic reorientation of energy-gaining mechanisms may be required for growth *in vitro* and that this process may be complicated by exceptional difficulty in transport of the elements required.

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VIRULENCE, TOXINOGENY, AND LYSOGENY IN *CORYNEBACTERIUM DIPHTHERIAE**

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About 15 years ago in Japan my associates and I developed an interest in certain unorthodox manifestations of the disease diphtheria. This led to a study of equally unorthodox diphtheria bacilli. Since then we have continued to explore the biology of *Corynebacterium diphtheriae*. This pursuit has been principally as (1) a study of properties of diphtheria bacilli that may be expressed in the animal host, for example, virulence and toxicity; and (2) an inquiry into the origin of these same attributes in the lysogenic, toxinogenic diphtheria bacillus.

Virulence and Toxinogeny in C. diphtheriae as Factors in Diphtherial Infections

From the time that Roux and Yersin (1888)[1] demonstrated that the diphtheria bacillus produces a soluble poison to the present, the idea has persisted that it is diphtherial toxin per se that enables *C. diphtheriae* to establish itself in animal hosts.[2] In fact this concept is so well embedded in bacteriological textbooks that the routine intracutaneous test for diphtherial toxin in the skin of the rabbit or the guinea pig is referred to as a "virulence test." It is, of course, not a test for virulence. It is an exquisitely sensitive method for the immunochemical detection of minute amounts of diphtherial toxin.[3] Virulent diphtheria bacilli are those that can become established and proliferate on mucous membranes and in the skin. Such organisms may or may not produce toxin. Since both toxinogenic and nontoxinogenic strains of *C. diphtheriae* can cause infections, it is obvious that the ability to infect, or invasiveness, and the ability to produce toxin are distinct. While this is true, nowhere in the literature of diphtheria is there available a record of a laboratory-contracted infection in humans where the effects of invasiveness could easily be distinguished from the effects of toxin and vice versa. Edward and Allison[4] have recorded cases of clinical diphtheria attributed to nontoxinogenic strains of *C. diphtheriae*. Over the past 15 years there have come to our attention two accidental infections and one accidental intoxication, the study of which provide us with models of invasiveness divorced from toxaemic manifestations and diphtheric toxaemia divorced from bacterial infection.

Virulence. The much studied[5-7] mitis (smooth) strain of *C. diphtheriae*, $C7_s(-)$, when introduced into the skin of rabbits, gives rise to a purulent lesion surrounded by an area of edema. Antitoxin has no effect on this reaction. Figure 1 is a photograph of the back of a rabbit on which have developed 11 lesions resulting from the injection of 7 pairs (14 injections) of strains of *C. diphtheriae*. At time zero different strains are injected at the sites 1 to 7. At

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time 3½ hours a dose of 1000 units of horse antitoxin is given intravenously to the rabbit. At 4 hours strains of diphtheria bacilli identical with those given initially are injected at the sites 1′ to 7′. These last injections serve as controls; without them the results are difficult to interpret. While we are con-

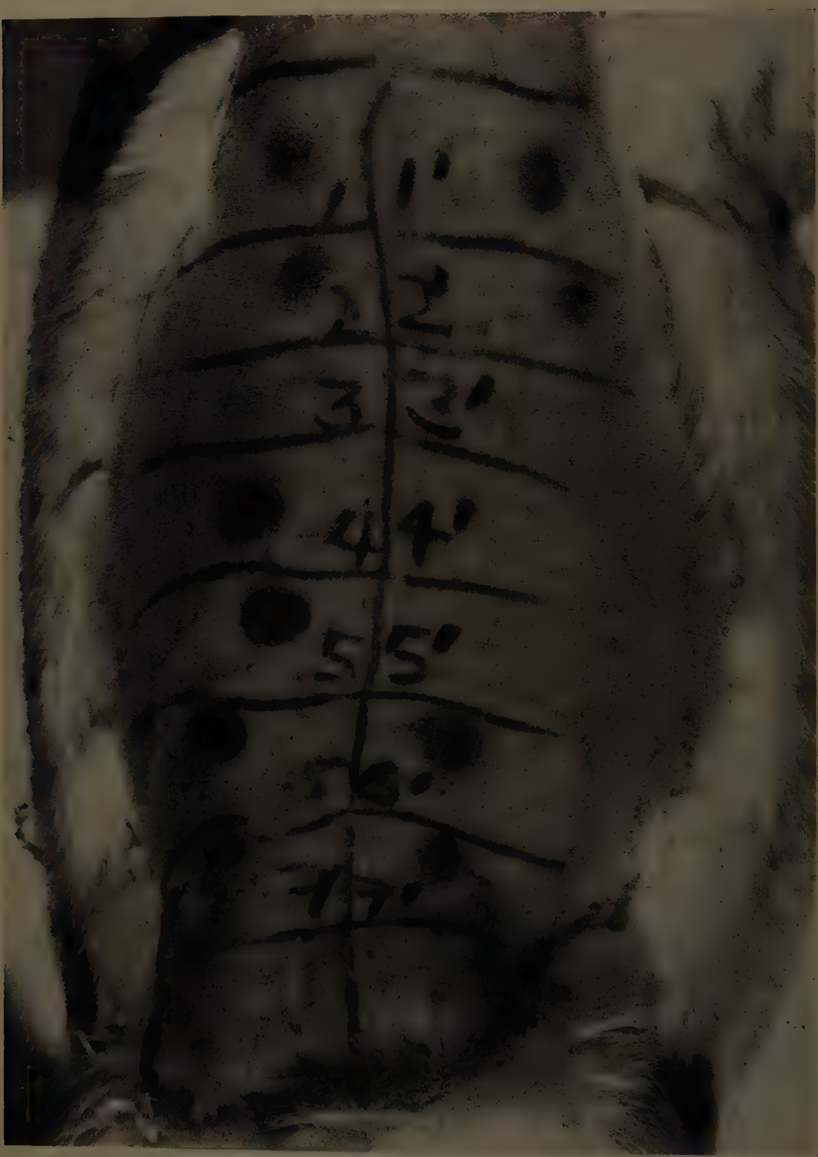

FIGURE 1. Lesions produced in the skin of a rabbit by virulent and nontoxinogenic, virulent and toxinogenic, avirulent and toxinogenic, and avirulent and nontoxinogenic strains of *C. diphtheriae*. For description see text. Note: the photograph has been inked in at sites 6 and 7 because of the amount of hair present over the necrotic portion of the lesion.

cerned at this moment only with the reaction given by strain $C7_s(-)$, sites 2 and 2′ in the figure, the intracutaneous test will be better understood if we stop to consider in turn each of the reaction sites. Sites 1 and 1′ represent responses to the injection of $C7_s(\gamma)$, a lysogenic, invasive nontoxinogenic diphtheria bacillus. At sites 2 and 2′ $C7_s(-)$, our model virulent or invasive strain[*] was injected. Site 3 is remarkable in that no reaction occurred as a result of injection either before or after the administration of antitoxic serum. This is the reaction to be expected from a completely innocuous diphtheria bacillus. Sites 3 and 3′ are occupied by $C8_r(-)$, a mutant of the Halifax strain *C. diphtheriae* gravis.[8] At site 4 is a typical necrotic plaque caused by diphtheria toxin alone or, in this case, as elaborated by a toxinogenic strain. At site 4′ there is no reaction. Antitoxin has neutralized the toxin introduced at site 4′ and the toxinogenic organism PW8(P), being less virulent, has failed to elicit an inflammatory response. Sites 5 and 5′ received another PW8 strain, PW8(Pd), which will be discussed later. Sites 6 and 6′ represent the reaction of the rabbit to a virulent, toxinogenic strain, $C7_s(\sigma)$. The necrotic plaque at site 6 is somewhat obscured; it is of course absent from site 6′. Site 7, far down on the rabbit's back, is a manifestation of a reaction to a virulent organism that produces very little toxin. I here call attention again to sites 2, 2′, 3 and 3′. The former pair illustrate the reaction elicited by a virulent organism that grows as a smooth colony, the latter by an avirulent bacillus that forms a rough colony. The surface antigen associated with the smooth colony is rich in lipid. Associated with the surface antigen are receptors for several of the corynebacterial phages (there are a few phages that attack rough *Corynebacteria*). These receptors are absent from the rough, avirulent strains. While much work has been done on the characterization of corynebacterial lipids, especially the mycolic acids,[10] we have no information to suggest what role lipoid components play in the virulence of *C. diphtheriae*.

It has been possible to follow two laboratory infections caused by the mitis strain of *C. diphtheriae* $C7_s(-)$. TABLE 1 is a resumé of one of these. The two infections were contracted several months apart. In one case, infection occurred following the accidental swallowing of perhaps one million organisms by a medical student in the midst of a pipetting operation; in the second, the bacilli were apparently inhaled by a graduate student who was making a cell wall preparation from millions of $C7_s(-)$ cells. Each of the individuals was immune to "diphtheria"; that is, they were Schick-negative. One had a circulating antitoxin level of over 0.5 antitoxin units per milliliter. Each developed a sore throat after an incubation period of 48 hours. In both cases about 12 hours later, 70 hours after exposure to the bacilli, small amounts of tenacious, greyish-white membranous exudate were observed on the tonsils, especially around the edges of the crypts.

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[*] The following is a report of a microscopic examination of lesions produced by $C7(-)$ in the skin of a rabbit. Two tenths of a milliliter of a fresh culture, optical density 3.0, was injected at each of 3 sites. Seventy hours later the animal was sacrificed and skin containing lesions was dissected out and placed in formalin. We are indebted to Norman S. Cooper, Department of Pathology, New York University College of Medicine, New York, N. Y., for the "pathology report":

"Block 1. A large mass of neutrophils, histiocytes, lymphocytes—largely necrotic—occupies the dermis interspersed with collagen bundles at the periphery, and in the underlying skeletal muscle, are well preserved acute inflammatory cells and slight granulation tissue. The skin is ulcerated and the surface layer consists of a scab-necrotic, dried inflammatory cells, epithelium.

"Block 2: Similar, but dermal abscess is more sharply circumscribed, perhaps more liquefied. No ulceration in section.

"Block 3: Similar to 1, with ulceration. There is no clear-cut scab, but there is a zone of 'necrotic' dermis at edge of ulcer."

In the case of the medical student the largest patch of pseudomembrane measured approximately 7 mm. in diameter when first noted. It was rubbed vigorously with a sterile swab to obtain material from which to prepare cultures and smears. Much of the exudate seemed dislodged by this treatment but 4 hours later more of it appeared at the same spot, this time covering an area of approximately 12 mm. in diameter. Gram-stained smears revealed numerous Gram-positive cocci, fusiform bacilli, and pleomorphic Gram-positive rods indistinguishable from *C. diphtheriae.* After overnight incubation, chocolate tellurite-plates seeded with exudate from the medical student's tonsil contained several hundred colonies of *C. diphtheriae* mitis. These were shown to be nontoxinogenic by the intracutaneous test; they were penicillin-sensitive and in every way were identical with the C7 strain. As may be seen from TABLE 1 it was possible to recover a few organisms from the student's throat during and following intensive penicillin therapy.

TABLE 1

INFECTION OF MEDICAL STUDENT WITH *C. DIPHTHERIAE MITIS.*, STRAIN $C7_s(-)$

12-17-56	Swallowed culture (approximately 1 million organisms at 4 P.M.).
12-18-56	Reported event of 12-17-56. Throat clean, of normal appearance. Swabbed for culture.
12-19-56	Five hundred to 700 colonies recovered from swab. Four isolated in p.c. and grown up for guinea pig toxinogenicity test.
12-20-56	T. developed sore throat during night of 12-19-56. Swabbed for culture → 500 to 700 colonies $C7_s(-)$ recovered. No exudate observed in throat.
12-21-56	Membranous exudate ±7 mm. surrounding shallow crypt on right tonsil. Small flecks of exudate at other points. Corynebacteria observed in gram-stained smear. Culture yields 400 to 500 colonies. Hospitalized. Penicillin therapy initiated.
12-22-56	Throat normal. 300 to 400 colonies of $C7_s(-)$ recovered.
12-23-56	Throat apparently normal. 5 colonies recovered from left tonsil; 150 from the right.
1-10-57	Two hundred to 300 colonies recovered from throat swab.
11-19-58	Two hundred to 300 colonies recovered from throat swab.

The graduate student was treated with achromycin (250 mg. every 4 hours for 4 days). He was not hospitalized. The amount of visible exudate on one of his tonsils was greater when first noted and persisted longer (24 hours) after the initiation of treatment than in the case of the medical student. Both men were asymptomatic 36 hours following the institution of antibiotic therapy. Diphtheria bacilli could, nevertheless, be cultivated from their throats during the period of antibiotic treatment. Both students harbored strain $C7_s(-)$ in their throats 1 year after the onset of these mild infections. One of them was still carrying the organism 2 years later.

Here then are cases of infection by virulent, nontoxinogenic diphtheria bacilli in the presence of circulating antitoxin, and the course of infection has been followed from time of contact through to the establishment of the carrier state. While these episodes were mild in the adult male, there is ample evidence[4,10] that such infection may lead to extensive pseudomembrane development in children.

Diphtheric toxaemia in children in the absence of infection. Diphtheria toxin is a well studied protein, M.W. close to 70,000. It is fairly toxic for animals: 0.06 μg toxin protein is lethal for guinea pigs of 250 gm. Toxin may be rendered into innocuous toxoid by treatment with formalin at alkaline *p*H. Toxoid is used the world over as an immunizing agent against diphtheria. In Japan in 1946 a failure occurred in the preparation of toxoid by a pharmaceutical company. Toxin prepared by this company from the Park-Williams number 8 strain of *C. diphtheriae*[11] of low titer (2 Lf) was divided into 4 batches, only 3 of which received formalin. All 4 were bottled and shipped out for use in a broad program of immunization against diphtheria, a disease then very prevalent among Japanese children. The result was a tragedy of the worst sort. About 700 children in the Kansai district of Japan were subsequently reported to have been adversely affected by the second of two 1-ml. doses of diphtherial toxoid administered one week apart in the course of the immunization program. One of us (L.B.) was called in to help determine the origin of the poisonous toxoid. At that time we (L.B. and K.H.) were studying toxin production by various strains of *C. diphtheriae* then prevalent in Japan[12] and so were able to evaluate quickly the "toxoid" involved in the Kansai tragedy.

Sixty-two children died as a direct result of the second immunizing dose. Through the cooperation of local health authorities, both Japanese and United States, it was possible to obtain case histories on 54 of the 62. A number of different physicians were responsible for the case histories and there were varying degrees of completeness from history to history. There emerges, nevertheless, the picture to be expected in diphtheritic toxaemia. Necrosis with ulceration at the site of inoculation was noted in 32 cases, local edema in 52, axillary lymphadenopathy in 7, pharyngeal paralysis as evidenced by difficulty in swallowing in 34, and urinary failure in 9. The causes of death among the 54 cases as reported by the attending physicians were: "heart failure and post diphtheritic paralysis," 18; "pneumonia," 10; "general emaciation," 2; "paralysis of the diaphragm and pharyngeal muscles," 7; and "cellulitis and ulceration of the upper left arm," 1.

The "toxoid" responsible for the death of these children was a sterile filtrate containing no detectable formalin; commercially prepared toxoid, obtained at that time from the United States, contained 0.17 mg./ml. formalin. As already mentioned, the amount of "toxoid" in the preparation was 4.88 μg./ml. or 2 flocculating units (Lf.). The number of Lf. was ascertained by blending with a toxin of known value. The amount of toxin found was 10 guinea pig minimal lethal doses.* The minimal lethal dose (MLD):Lf. ratio in toxin varies from 35 to 40 per Lf. unit. Rarely, even in a very fresh toxin, is a ratio of 50:1 found. Under suitable conditions, crude toxin undergoes spontaneous conversion to toxoid on long standing.[13] Appreciable spontaneous conversion must have occurred in the preparation under consideration, since it contained only 5 MLD per flocculating unit. Because of this, a number of the 700 children reported to have been inoculated with it were spared its fatal effect.

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* The MLD of Ehrlich is defined as the least amount of toxin that will cause the death of a 250-gm. guinea pig in 4 days. Here we employed 6 guinea pigs, 250 gm., for each dilution of toxoid and determined the LD_{50} according to the method of Reed and Muench.[15]

Most of the 62 children succumbing to the toxin died after the fourth day. Their deaths were spread out over a 45-day period, the greatest number dying around the 35th day. As a result of this observation, a record was kept of the guinea pigs surviving the fourth day but dying subsequently, since what we were really interested in was the lethal effect of the tested "toxoid" irrespective of time. In FIGURE 2 the lethal times for the affected children and for the test animals are plotted. Note that 0.04 ml. of toxoid served as an LD_{50} for guinea pigs when the lethal time was extended to thirty days. Were the sensitivity of infants comparable to that of guinea pigs, the lethal dose for a 10-kg. child* should have been 1.6 ml. (10/0.25 × 0.04) since the weight of the average child was 40 times that of the guinea pig. Actually, 1.0 ml. (10 standard guinea pig MLD) or less was a lethal dose for the children. The reason for saying "or less" is that each child had received, one week before the fatal dose, an injection of 1 ml. of toxoid. This may have had some, though slight, effect upon the immune status of the children.

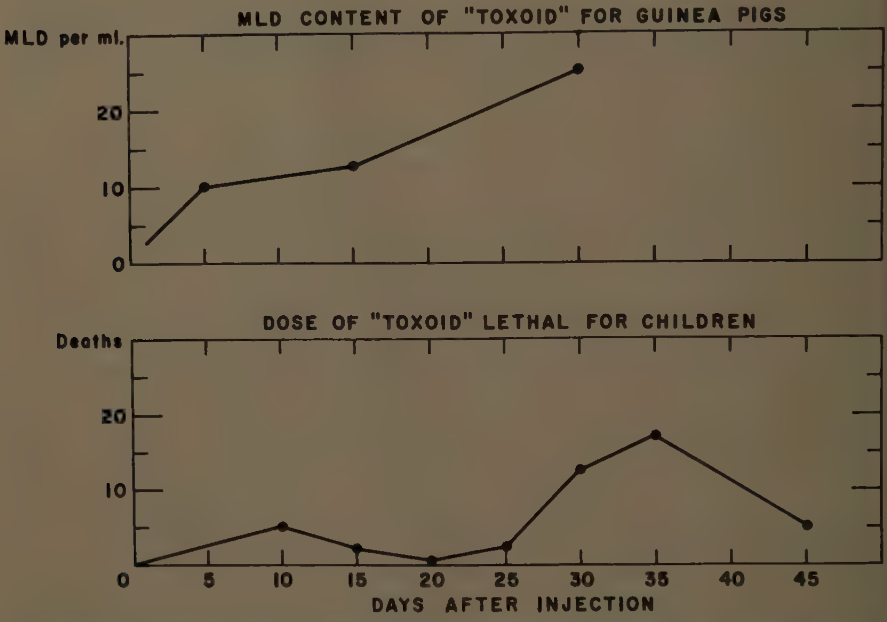

FIGURE 2. A comparison of the lethal effects of diphtherial toxin upon guinea pigs and upon human infants. "Toxoid" was actually toxin, 88 per cent of which had spontaneously undergone conversion to toxoid.

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* Weights were not recorded by the physicians attending these children, therefore the weights were ascertained by noting the sex and age of each child and computing the weight from a table of weights in a standard Japanese textbook of pediatrics.

Lysogeny and Toxinogeny

When a strain of *C. diphtheriae* such as $C7_s(-)$ is infected with the temperate phage β, a stable lysogenic, toxinogenic organism $C7_s(\beta)$ results.

$$C7_s(-) + \beta \text{ phage} \rightarrow C7_s(\beta)$$

sensitive to β phage, virulent, nontoxinogenic, nonlysogenic for β phage	resistant to β phage, virulent, toxinogenic, lysogenic for β phage

$C7_s(\beta)$ and all of its progeny carry β-phage in a noninfectious form called prophage. Prophage is that form of phage perpetuated in lysogenic bacteria. All cells carrying β-prophage are toxinogenic. When β-prophage is eliminated from $C7_s(\beta)$, a nonlysogenic, nontoxinogenic, phage-sensitive bacillus comes into being.[14] Reinfection of $C7_s(-)$ restores the lysogenic, toxinogenic state. β-prophage, then, is the genetic determinant of lysogeny and toxinogeny.

In a lysogenic population of *C. diphtheriae* one cell in 50,000 lyses spontaneously and releases phage. If the cells are starved, denied adequate oxygen, or mistreated in a number of ways including exposure to certain doses of ultraviolet light, X rays, hydrogen peroxide, nitrogen mustard, the rate of lysis is much enhanced. Lysis brought about by specific agents is called "induction."[16] These agents induce prophage to enter the vegetative state and this leads to phage multiplication with destruction of the host cell. In FIGURE 3, taken from one of our early experiments,[7] is shown the way in which the viability of an ultraviolet light-induced culture of *C. diphtheriae* drops as lysis occurs and phage is liberated.

When in the life of the diphtheria bacillus is toxin produced? Diphtherial toxin has long been regarded as one of those emergent aspects of growth that are termed, for example, extracellular products and exotoxin. At one time bacteriophage itself was said to be such an extracellular product, and it could be shown for *Bacillus megaterium* at least that the product (termed a gelatinase) increased exponentially with time in an actively growing culture.[17] Most investigators agree, however, that diphtheria toxin is not produced in actively growing cultures. It makes its appearance at the end of growth.[6,18,19] We rather think that toxin is a product of a dying cell and that the cell is dying a particular kind of death resulting from the multiplication of bacteriophage.

Today there is no question about the fact that diphtherial toxin can be produced only in culture media low in iron. It is also known that toxin can be produced only under aerobic conditions, and it follows that aerobic organisms have a need for iron. When these organisms run out of iron their capacity to synthesize cytochromes, catalase, and other haem enzymes become diminished. At one time, we reasoned that as catalase levels fall in iron-starved *C. diphtheriae*, enough peroxide might accumulate to induce the carried prophage to become vegetative phage and cause the lysis of the cell. In FIGURE 4 data are plotted from an experiment in which a logarithmically growing culture of *C. diphtheriae*, SM-1 of Yoneda,* is washed, resuspended in fresh iron-deficient medium, and allowed to run out of iron under conditions of vigorous aeration. The release of phage and toxin begins after growth slows down. The amount of toxin is small and we know now that the SM-1 strain was in fact a mixed culture of two wonderful organisms: PW8(P) and PW8(Pd). PW8(P) has a division time of 90 min., PW8(Pd) 155 min. The time from induction to lysis in *C. diphtheriae* is twice the division time. Thus most of the phage and most of the toxin seen here probably comes from the PW8(P) strain. If induction is really all that is required to initiate the synthesis of toxin, it should be possible to substitute ultraviolet light for iron deficiency as the triggering mecha-

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* We received the SM-1 strain as a pellicle culture (surface film in liquid medium). Plating on chocolate agar revealed two colonial types: PW8(P), which requires tryptophan for growth, and PW8(Pd), a tryptophan-independent strain. This mutation from PW8(Pd) to PW8(P) is of no consequence in the experiments of Yoneda since he always prepares toxin in a tryptophan-deficient medium.[25]

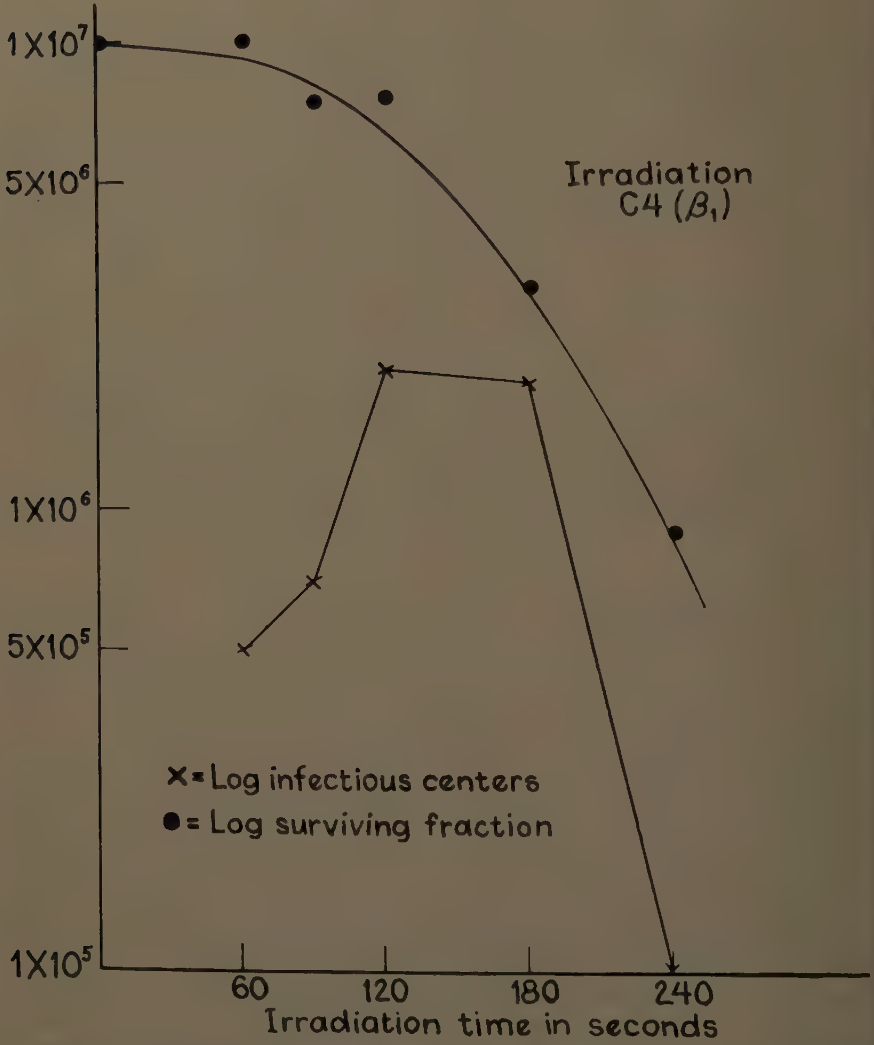

FIGURE 3. Induction of β-prophage with concomitant death of C4(β) following irradiation with ultraviolet light.

nism. In TABLE 2 the results of an experiment are shown in which strain PW8(Pd) was washed, resuspended in media of various iron levels, irradiated, and, at the end of 300 min. assayed for toxin. Nonirradiated controls were run for each iron level. Two things are evident from this experiment: ultraviolet light enhances toxin yields as much as 15-fold, but only at low iron levels. This suggests that induction is indeed important to toxinogenesis but that sufficient, if somewhat unphysiological, levels of iron can suppress some process or processes between induction and toxin synthesis.

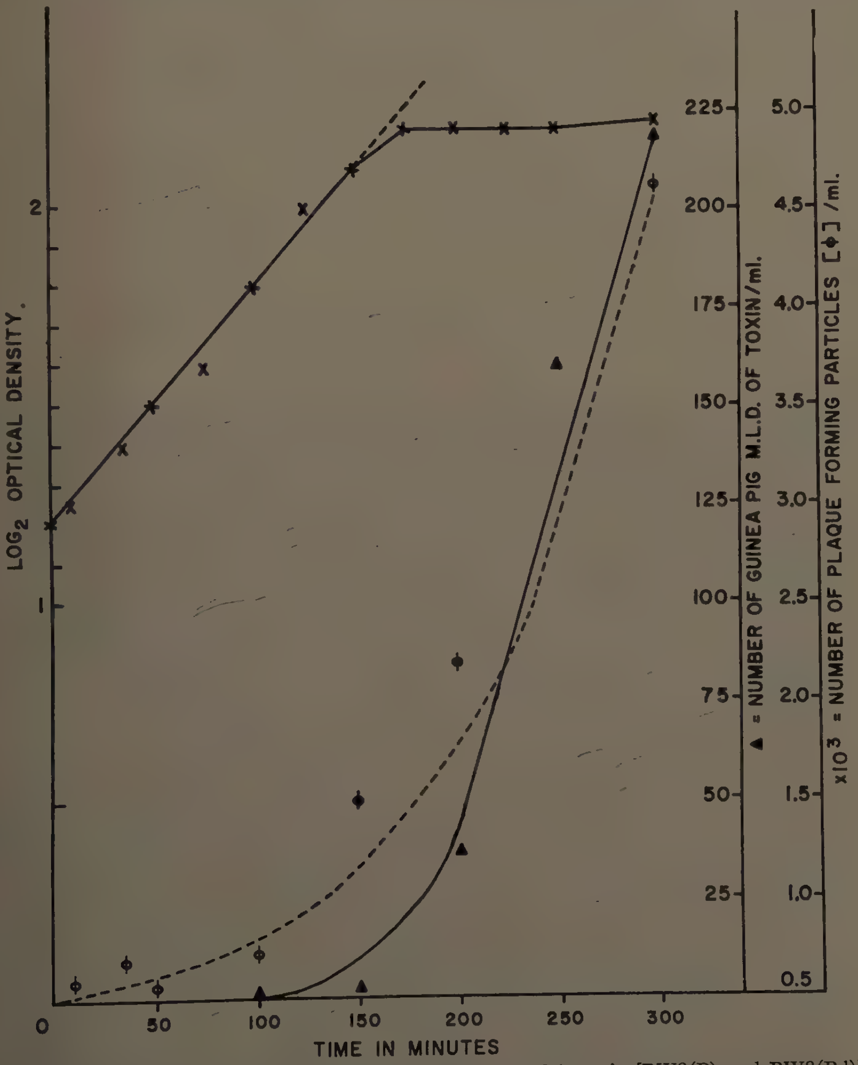

FIGURE 4. The release of toxin and phage by the SM-1 strain [PW8(P) and PW8(Pd)] grown in a medium containing limiting quantities of iron. Cells were washed, suspended in deferrated medium, and samples were taken at the indicated times for the measurement of optical density, free phage, and toxin.

Induction, as we have noted, leads to phage multiplication. If phage multiplication is all that is needed for toxin production, it should be possible to make toxin by simply lysing diphtheria bacilli with a lytic toxinogenic phage. This is possible. Phage B, a lytic mutant of β, will lyse the nontoxinogenic strain of *C. diphtheriae* $C4_s(-)$. The amount of toxin so produced is small but enough to be detected by the rabbit intracutaneous test. A study of B phage-$C4_s(-)$ system indicates that the conditions required for getting the phage into the cell are inimical to the production of toxin. Furthermore, all of the indicator strains available are smooth organisms, and something about smooth organisms, probably the amount of lipoid material they produce, tends to inhibit the production of toxin. In TABLE 3 are listed four lysogenic *C. diphtheriae* each carrying the right phage for toxinogeny. One is an abnormally smooth organism designated SSSmooth, a second $C7_s(\beta)$ has been designated SSmooth and in a like manner to rough.

TABLE 2

THE EFFECT OF ULTRAVIOLET IRRADIATION UPON TOXIN PRODUCTION BY THE PW8(PD) STRAIN GROWN IN MEDIA DIFFERING IN IRON CONCENTRATION

Iron (γ/ml.)	Culture*	Toxin (MLD)
1.0	A	>1
	B	>1
0.75	A	>1
	B	>1
0.55	A	2
	B	10
0.1	A	10
	B	50
0.075	A	10
	B	120

* Optical density of culture: 3.0 ≈ 3.6 × 10^9 bacteria/ml. B = half of culture irradiated with inducing dose of ultraviolet light (180 secs. at 76 cm.). A = nonirradiated control. 1 MLD ≈ ±40 Lf.; 1 Lf. = 2.4 μg. toxin protein.

There is no doubt that smoothness, which is correlated with virulence, does not correlate with maximal toxin production (TABLE 3). This is borne out by the fact that mitis strains (associated with mild clinical disease) are invariably smooth.*

FIGURE 5, which is a photograph of PW8(P) and PW8(Pd) growing on the same plate, affords an idea of how a small change in a surface component affects the way in which the bacterial cells pile up to form a colony. These are two very valuable toxinogenic bacilli. PW8(Pd) carries a defective phage, one that harbors a genetic block that prevents the formation of mature phage.

* In disease, the diphtheria bacillus, to produce the most severe symptoms, must be both invasive and toxinogenic. This condition is realized in gravis organisms such as $C8_s(\phi d)$, which are intermediate between a smooth strain[21] and a rough strain and which yield more toxin than mitis strains.

The existence of strain PW8(Pd), then, means that while induction is necessary for toxinogenesis, phage maturation is not. PW8(P) is valuable because, though it makes less toxin than PW8(Pd), it liberates mature phage, thus providing a means for following the release of phage along with that of toxin.

In the induction experiments considered earlier, the amount of toxin released concomitant with phage was rather small: 240 MLD or so (about 14 μg. toxin protein per milliliter of filtrate). It turns out that the experiments were termi-

TABLE 3

Strain	Maximal recorded toxin* yields
SSSmooth $C8_s$(B)	<0.0025 μg./ml.
SSmooth $C7_s$(β)	32 μg./ml.[6]
Smooth PW8(P)	96 μg./ml.
Rough PW8(Pdi)	360 μg./ml.[6]

* Toxin protein.

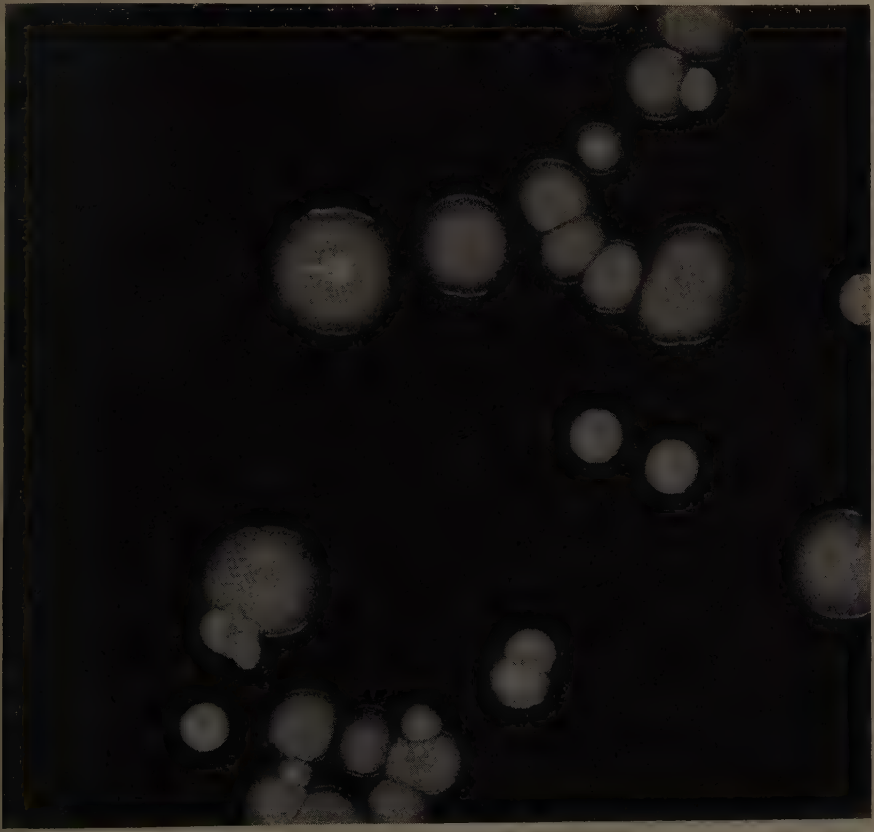

FIGURE 5. *C. diphtheriae* SM-1 strain of Yoneda[20] plated on chocolate agar. Small colonies are PW8(P); large colonies are PW8(Pd).

nated too soon. For example, if a culture of the PW8(Pd) strain is irradiated and the end of latent period is awaited for the appearance of toxin, the yield is about 20 μg. If, however, samples continue to be taken for a time equivalent to one or more latent period, 100 μg. or more of toxin is formed.[21]

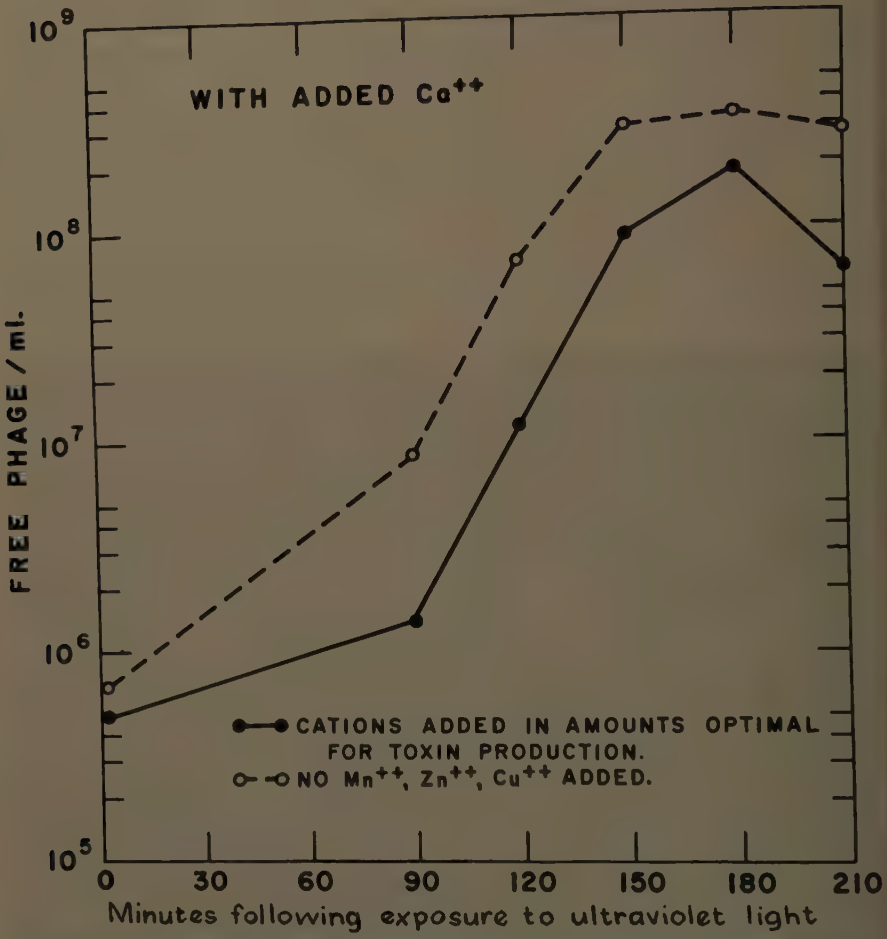

FIGURE 6. The effect of added Mn^{++}, Zn^{++}, and Cu^{++} on the kinetics of P-phage release O.D. ≈ 0.3. Inducing dose: 110 sec. at 76 cm. Calcium chloride 10^{-3} M. Strain PW8(P)

The medium used by us and many others[22-26] for toxin production is that of Mueller and Miller.[27] It is a casein hydrolysate broth to which are added large amounts of calcium, magnesium, zinc, manganese, and copper. The latter three are not needed in such excessive amounts for the growth of *C. diphtheriae*, and they have an interesting effect on phage yields. For example, the omission of all three (Zn^{++}, Mn^{++}, and Cu^{++}) allows much higher yields of phage. This is clearly demonstrated in FIGURE 6 where equal numbers of cells grown in the presence and absence of these cations are induced with ultraviolet light and phage liberation followed. Mueller and Miller's medium also contains much calcium. When this is not provided (FIGURE 7), the difference in the yields of phage in the presence and in the absence of added zinc, manganese, and copper is even greater. It is interesting that iron also affects the amount of phage produced (FIGURE 8). The medium found by many workers to be

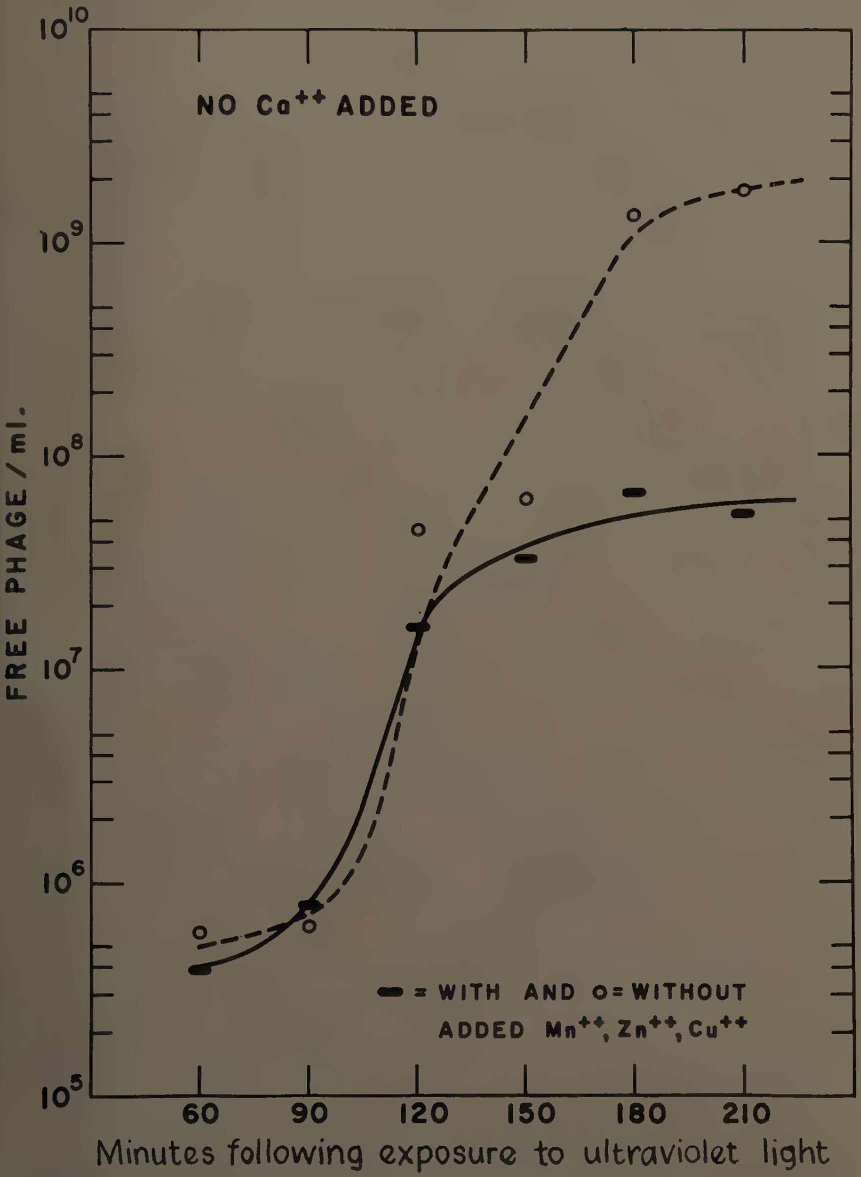

FIGURE 7. The effect of added Mn^{++}, Zn^{++}, and Cu^{++} on the kinetics of P-phage release. Conditions as in FIGURE 5, but no calcium chloride added. Strain PW8(P).

optimal for toxin production, then, tends to suppress phage liberation. This fact, in connection with the other data presented here, suggests that in order to make toxin the diphtherial cell must have its inherent phage machinery set in operation, but that the shorter the period of operation the smaller the size of the yield of toxin. Since the process of phage liberation lyses the cell, it dis-

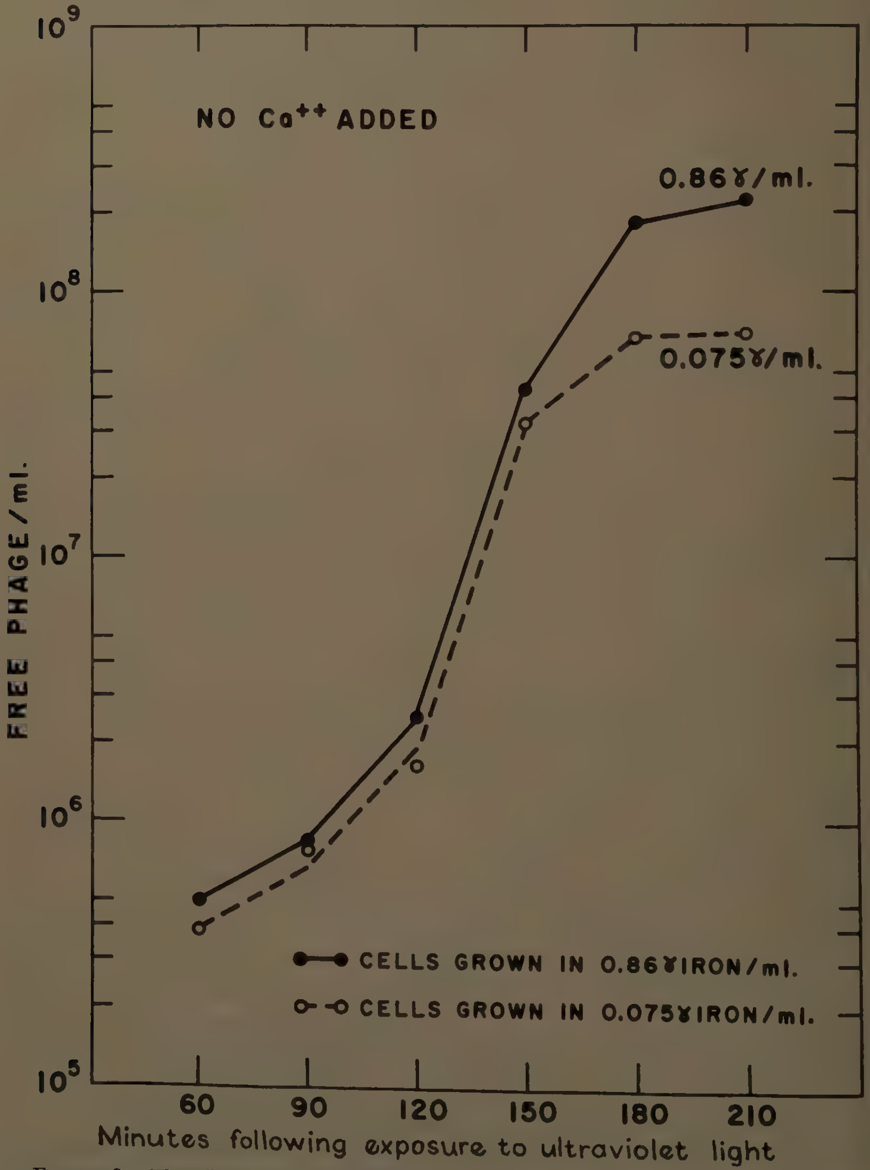

FIGURE 8. The effect of iron concentration on the kinetics of release of P-phage by strain PW8(P) grown in medium to which no calcium chloride has been added.

rupts the toxin-synthesizing system. As a kind of test of this hypothesis let us consider the effect upon toxin yields of an agent that delays lysis of the induced diphtheria bacillus: proflavine. In TABLE 4 it may be seen that if proflavine is added before induction, very little toxin is formed. If no proflavine is added, the time to lysis in this experiment is 250 min. If proflavine is added at 100 min. after induction, the time to lysis is increased 33 per cent, the phage yield is decreased fivefold, and the toxin yield is increased slightly more than twofold.

Summary and Conclusions

A brief survey of the biology of the diphtheria bacillus has been presented. Particular attention has been paid to its virulence (invasiveness) and to its toxinogeny. The former property is under the genetic control of the bacterium, but the gene governing toxinogeny resides in certain bacteriophages. Virulent, nontoxinogenic *C. diphtheriae* become virulent, toxinogenic bacilli following infection with one of these phages.

TABLE 4
THE EFFECT OF PROFLAVINE ON PHAGE AND TOXIN YIELDS AND ON THE TIME OF LYSIS OF STRAIN SM-1 INDUCED WITH ULTRAVIOLET LIGHT*

Time (min.) of adding proflavine†	Time of lysis	Phage titer	Toxin (MLD)†
−10	260	3×10^4	22
Inducing dose of U.V. at zero time			
50	370	1.6×10^5	225
100	320	4×10^6	245
No proflavine added	250	2.5×10^7	120

* O.D. ≈ 2.7 ≈ 3.2×10^9. Bacteria per milliliter ± 40 MLD ≈ 1 Lf. ≈ 2.44 μg. toxin protein.

† Eight μg./ml.

The production of toxin requires the activation of the phage-multiplying machinery latent in the lysogenic toxinogenic strains. Rough bacilli are avirulent and good toxin producers. Smooth bacilli are virulent and give poor yields of toxin. The property of virulence, then, is associated with smoothness.

Toxin yields are enhanced by certain agents that delay the onset of lysis, that is, lengthen the latent period. An ideal organism for use in a study of the kinetics of toxin formation would be a rough (nonsmooth), inducible, toxinogenic diphtheria bacillus characterized by a long division time. This latter property would assure an increased latent period. Such a diphtheria bacillus is available. It is the PW8 strain, discovered by William Hallock Park in 1896.[12] We are slowly becoming aware of how well suited it is for making a large quantity of diphtherial toxin.

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ORAL POISONING BY BACTERIAL EXOTOXINS EXEMPLIFIED IN BOTULISM*

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In botulism the harm done to the animal victim is the result of a toxemia following ingestion of poisoned food and does not involve an infectious process. The goals of biochemistry are to relate the toxemia to the chemical structure of the toxin molecule and to identify those factors of susceptibility of the host to the toxin that have a biochemical basis (Lamanna, 1959).

Botulinal toxins appear to be simple proteins. It is not possible to say how the property of toxicity arises in this protein. The search for toxophoric groups within the structure of the protein molecule has been negative to date. A variety of unrelated physical agents and chemical reactions cause loss of toxicity. A recent hope that fluorescence of the toxin at 3300 Å after activation at 2900 Å is associated with toxicity (Boroff and Fitzgerald, 1958) has not been substantiated since detoxification can be accomplished without an accompanying loss of fluorescence (Schantz *et al.*, 1960). The available facts do not prove, but do support, a hypothesis that attributes toxicity to the maintenance of the structural integrity of the protein molecule as a whole.

The question of the existence of a separable toxophore within the protein can be approached indirectly by asking what the size is of the toxic particle passing the intestinal barrier. Since in the natural situation botulism results from food poisoning, it might be hoped that nature would be economical in means and permit only the toxicologically active fragment of the protein particle to escape from the intestine into the general circulation. Such a possibility would be reinforced by any normal tendency of the alimentary tract's permeability barriers to refuse passage to whole protein. Heckly *et al.* (1960) have studied this question and found the systemically absorbed toxin to have the dimensions of a protein. By ultracentrifugal analysis, the sedimentation value (S_{20}) of the toxin appearing in the lymph draining from the small intestine of the rat was found to be 7.9 ± 3.5. Again defeat has followed an attempt to find toxicity resident in a particle size smaller than the dimensions of a protein.

Alone among the classical bacterial exotoxins, botulinal toxin has been considered to be an oral poison. Exploration of this property might provide clues of a biochemical nature to the biological properties of the toxin. Unfortunately, I have recently shown oral toxicity not to be a characteristic unique to botulinal toxin (Lamanna, 1960). Both diphtheria and tetanus toxins can act as oral poisons and at the very most are only one order of magnitude less toxic orally than botulinal toxin in terms of the number of intraperitoneal LD_{50} doses required for one oral LD_{50} dose (TABLE 1). Oral toxicity of the bacterial exotoxins does not seem to be an expression of intrinsic qualities of chemical struc-

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ture of the toxins, but rather is a consequence of a physiological fact. This fact is the inability of the alimentary tract of the "normal" animal to prevent the escape of small quantities of whole proteins into the general circulation by way of the lymphatic route. The prime avenue of escape is the small intestine. This organ cannot be looked upon as an absolute barrier to the systemic absorption of whole protein, a fact that allergists have long recognized (Ratner and Gruehl, 1934). Potentially, any toxic protein is an oral poison if its potency is high enough for the minute amounts crossing the intestinal wall to exceed the threshold values for physiological activity at locations distant from the intestine.

The suspicion that botulinal toxin is without unique chemical attributes making for its oral toxicity is reinforced by the finding of the capacity of proteolytic enzymes of the small intestine to detoxify crystalline type A toxin (Meyer and Lamanna, 1959). While the rate of destruction in the intestine is apparently too slow to prevent a fatal dose from being absorbed, this slow rate of proteolysis can hardly be claimed to be unusual for a protein in the native state. Type E botulinal toxin can be activated by trypsin but the activated toxin is itself detoxifiable by trypsin (Duff *et al.*, 1956).

TABLE 1

NUMBER OF INTRAPERITONEAL LD_{50} REQUIRED FOR ONE ORAL LD_{50}*

Species	Toxin	
Mouse, 20 gm.	Botulinal type A	50,000 to 250,000
Mouse, 20 gm.	Tetanus	80,000 to 1,200,000
Guinea pig, 900 gm.	Diphtheria	100,000

* With the mice the toxins were administered orally by the use of a slightly curved blunt-nosed needle on a syringe. Diphtheria toxin was administered by forced feeding of gelatin capsules containing concentrated toxin. These methods appear to successfully introduce toxin into the gut without contamination of the mouth and throat and appear to be well tolerated without evidence of tissue trauma. Deaths, therefore, are thought to be truly representative of absorption of toxin from the normal gut.

The fate of the animal swallowing botulinal toxin is dependent not only on how much toxin is ingested but also on the state of alimentation and the kinds of foods being consumed (Lamanna and Meyers, 1960). Foods may act to increase or decrease oral toxicity of a fixed quantity of ingested toxin. Insufficient data are at hand to permit generalization and prediction of how particular kinds of foods will act. However, four possibilities worthy of investigation are self evident: foods might protect toxin against destructive intestinal influences, they might combine with the toxin to form larger particles less able to penetrate the gut wall than is free toxin, they might modify the physiological bases of intestinal permeability, or they might affect the rate of peristalsis with a consequent decrease or increase in the sojourn of the toxin in that part of the intestine offering the maximum opportunity for systemic absorption.

I suspect the effect of food is less on the toxin than on mechanisms influencing the permeability of the small intestine to whole protein. If foods did interfere with detoxifying proteolysis in the gut, it might be possible to show an enhancement of oral toxicity by the use of specific trypsin inhibitors. Soybean and egg albumin trypsin inhibitors have been tried and both fail by their presence in the alimentary tract to affect the oral toxicity of the tetanus and crystalline botulinal toxins under the test conditions (TABLE 2). The total number of mice succumbing to varying doses of orally administered toxin was not found to be significantly different in the presence and absence of the trypsin inhibitor when a twofold dilution series of toxin was employed at dilutions somewhat above and below the oral LD_{50} dose. The inability of trypsin inhibitor to increase oral potency of the toxin was disappointing, since it would be guessed that a large portion of the great disparity between the parenteral and oral lethal doses was the result of destructive proteolysis in the gut.

TABLE 2

NUMBER OF MICE SUCCUMBING TO ORALLY ADMINISTERED CRYSTALLINE TYPE A BOTULINAL TOXIN IN THE PRESENCE AND ABSENCE OF ORALLY ADMINISTERED TRYPSIN INHIBITOR

Experiment		1		1	2	3		1	2		1
						Soybean trypsin inhibitor					
Inhibitor present per mouse	0.2 mg.	11/32*	0.5 mg.	3/12	2/12	9/20	1 mg.	23/32	9/32	1.5 mg.	12/32
Inhibitor absent		12/32		3/12	4/12	11/20		25/32	8/32		13/32
						Eggwhite trypsin inhibitor					
Inhibitor present per mouse	0.5 mg.	8/32	1 mg.	22/32	10/32	1/32	2 mg.	6/32			
Inhibitor absent		11/32		21/32	8/32	4/32		8/32			

* Dead mice/number tested.

TABLE 3

LD_{50} OF MIXTURE OF SOYBEAN TRYPSIN INHIBITOR AND CRYSTALLINE TYPE A BOTULINAL TOXIN BEFORE AND AFTER LOW SPEED CENTRIFUGATION

		LD_{50}/ml.
Mixture of toxin plus inhibitor	Before centrifugation	1,600,000
	Supernatant after centrifugation	600,000
Control: toxin alone centrifuged as above mixture was		1,600,000

With soybean trypsin inhibitor it is a simple matter to demonstrate *in vitro* that a reaction occurs with botulinal toxin reducing the solubility of the toxin (TABLE 3). How this phenomenon may be involved in the picture obtained with ingested mixtures of inhibitor and toxin should be the subject of future studies. Similar findings were not obtained with egg white trypsin inhibitor.

Virulence, or the opportunity for harm to befall the host, is a relative matter arising from the interaction of host factors and the pathogenic agent. In botulism the biochemical substrate of the peripheral nervous system of the host affected by the toxin may be in quantity independent of the body weight of the poisoned animal. This statement is based on experience with mice (Lamanna *et al.*, 1955). The lack of a relationship between body weight and the quantity of toxin required for a fatal parenteral dose is not peculiar to botulism. I have found the same fact to be true for tetanus toxin (TABLE 4). Similar reports exist for *Shigella paradysenteriae* endotoxin in mice (Zahl *et al.*, 1943) α-naphthyl-thiourea in rats (Rall and North, 1953), and histamine in mice (Angelakos, 1960).

The experience with a parenteral route of injection cannot be generalized to include the oral route. With type A crystalline botulinal toxin and tetanus toxin that has been tried, the youthful mouse requires more toxin than does the older, heavier mouse for a lethal dose (TABLES 5 and 6). While common sense might rebel at such a finding, a fact of anatomy may come to our rescue. In length the small intestine averages 40 cm. in the young 12 to 14 gm. mouse, and 57 cm. in the old 40 to 43 gm. mouse. There is, roughly speaking, 50 per cent more intestinal surface area for the systemic absorption of toxin in the large than in the small mouse under comparison. Since I have already shown that the small and large mouse require the same minimum parenteral dose for lethality, the lesser oral dose for the large mouse could merely reflect the greater opportunity for systemic absorption before peristalsis removes ingested toxin from the bounds of the small intestine.

TABLE 4

INTRAPERITONEAL LD_{50} OF TETANUS TOXIN SOLUTION WITH DIFFERENT WEIGHT MICE*

Average weight		Technician	LD_{50}	
Small mice (gm.)	Large mice (gm.)		Small mice	Large mice
7.6	39.3	A	1,631,000	1,350,000
9	40	A	189,000	244,000
9.7	40.1	A	364,000	283,000
9.4	39.6	A	389,000	305,000
11	39	A B	111,000 81,000	55,000 53,000
9.4	37	A B	257,000 323,000	259,000 212,000

* The titration values for the small and large mice are not significantly different, the variation being within the limits of experimental error. The method of Pizzi (1950), which permits the use of the Reed and Muench type of calculation, was employed for determining the standard error of the LD_{50}.

Concluding Statement

No evidence has been developed for pinpointing the chemical basis for toxicity, both parenteral and oral, in botulism. Oral toxicity for botulinal toxin can hardly be considered an unusual property because diphtheria and tetanus toxins, bacterial exotoxins not ordinarily thought of as oral poisons, will cause toxemia when ingested. Perhaps in diphtheria this fact has some role to play in the natural infection since the organisms growing in the nasopharyngeal area are producing toxin that must in part be ingested as an inevitable consequence of the swallowing reflex. Thought should be given to the possibility that some cases of cryptogenic tetanus may arise from oral or intestinal poisoning.

TABLE 5

TITRATIONS BY THE ORAL ROUTE OF CRYSTALLINE BOTULINAL TYPE A TOXIN IN MICE OF DIFFERENT WEIGHTS

Toxin (ml.)	Weight of mice (grams)									
	Experiment 1 ♂		Experiment 2 ♀		Experiment 3 ♂			Experiment 4 ♂		
	13–15	38–40	12–14	40–43	13–14	20–22	37–39	13–14	20–22	34–36
0.08					20/20	17/20		12/20	15/20	
0.04	19/20*	20/20			17/20	13/20	17/20	5/20	9/20	13/20
0.02	2/20	20/20	7/20	16/20	2/20	8/20	11/20	1/20	1/20	11/20
0.01	1/20	9/20	1/20	15/20	8/20	5/20	8/20	0/20	2/20	10/20
0.005	2/20	5/20	3/20	7/20	1/20	3/20	7/20	1/20	1/20	0/20
0.0025	1/20	3/20	1/20	3/20			2/20			0/20
0.00125			0/20	2/20						

* Dead mice/number injected. The difference in deaths between the 13–14 gm. and 20–22 gm. mice is probably not significant. On the other hand, the LD_{50} for the largest mice is significantly less (less toxin required for death) than for the smaller mice.

TABLE 6

ORAL TOXICITY OF TETANUS TOXIN FOR MALE MICE OF DIFFERENT WEIGHTS

Toxin (ml.)	Weight of mice (grams)					
	Experiment 1		Experiment 2		Experiment 3	
	13–14	37–39	13–14	38–40	13–14	34–35
0.75			2/12	10/12	4/12	8/12
0.50	5/12*	6/12	2/12	4/12	5/12	3/12
0.25	2/12	1/12	0/12	3/12	1/12	4/12
0.125	0/12	1/12	0/12	4/12	0/12	2/12
0.0625	0/12	2/12				
0.03125	0/12	0/12				

* Dead mice/number injected.

Oral toxicity of botulinal toxin would appear to be affected by any factor that can influence the length of residence of active toxin in the small intestine and the permeability of the small intestine to whole protein. Our knowledge of these problems is still at the stage of development of fundamental descriptive data. In the immediate future we will require knowledge of the anatomical and physiological bases for these data as they are acquired before we can proceed to the ultimate biochemical understanding of toxicity at the molecular level.

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BIOCHEMICAL PROPERTIES OF VIRULENT AND AVIRULENT STAPHYLOCOCCI

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A familiar concept in the host-parasite relationship defines the outcome of this relationship in terms of the balance struck between two opposing forces: the virulence of the invader on one hand and the resistance of the invaded on the other. In the case of staphylococcus as parasite and man as host, this concept suffers somewhat from the fact that we are unable to measure either of the quantities involved. At the present time there is no adequate method for measuring degrees of virulence among strains of staphylococci nor are there reliable means for assessing the degree of resistance in a given individual. Nevertheless when staphylococcal parasitism progresses to human disease, the event is often explained by postulating a strain of increased virulence, a weakened host, or both.

As long as we persist in considering two unknowns together, it seems unlikely that we shall ever fully understand either term in the host-parasite equation. If we wish to hold one term constant, which shall it be? There is some evidence for variable resistance: for example, the association of diabetes and furunculosis in the same individual is more frequent than chance would predict. However this example is probably overworked. Most staphylococcal infections occur in individuals who are not diabetic and who show no other evidence of altered metabolism. It would therefore seem to be more profitable to assume that individuals differ very little in their susceptibility to staphylococcal infection and to focus attention on virulence in the parasite.

The first assignment here, then, will be to attempt to show that strains of increased virulence do exist among staphylococci and, indeed, that these strains account for the majority of true staphylococcal infections in man. Since virulence is undoubtedly but one expression of microbial metabolism, it is reasonable to examine biochemical properties of many strains in seeking a clue to virulence.

All staphylococci that we have tested have had the following characteristics in common: all have been catalase-positive, have produced peroxidase in alkaline media, required thiamin and niacin for growth, have grown well on the surface of agar media containing 10 per cent sodium chloride, and have produced acid without gas from glucose, maltose, sucrose, and glycerol. These, together with their more obvious characteristics such as cluster formation of gram-positive cocci and growth in ordinary media both aerobically and anaerobically, are the properties that unite the staphylococci. Beyond these properties there are very many reactions by which individual cultures may be found to differ from each other. Thus there is an almost limitless number of physiological strains possible.

Among the properties that are not shared by all staphylococci, are there some which are related to virulence? This subject has been examined in detail elsewhere[1,2] and has been well summarized, notably in the reviews of

Blair[3,4] and in the recent and excellent monograph by Elek.[5] A list of some of the properties that have been found more frequently in virulent than in avirulent staphylococci is shown in TABLE 1. To this list we might add two observations of our own, namely that fermentation of d-ribose correlates well with mannitol fermentation, and that most virulent strains produce RNase as well as DNase.

For most of the properties listed in TABLE 1 it is possible to construct teleological arguments to show that possession of a particular toxin, enzyme, or other property may be useful to an organism in establishing or maintaining infection. Coagulase production is the property that has perhaps had the most success in this respect. Coagulase testing, furthermore, has assumed prominence as the favored single method for distinguishing between pathogenic and nonpathogenic staphylococci.

TABLE 1

SOME PROPERTIES THAT HAVE BEEN FOUND MORE FREQUENTLY IN VIRULENT THAN IN AVIRULENT STAPHYLOCOCCI

Enterotoxin
Dermonecrotoxin
Alpha and delta hemolysins
Fibrinolysin
Coagulase
Leucocidins
Hyaluronidase
Deoxyribonuclease
Phosphatase
Lipase
Pigment
Gelatinase
Mannitol fermentation
Reduction of tellurite and tetrazolium salts
Growth in normal human serum
Growth in the presence of certain basic dyes

Undoubtedly, most virulent staphylococci are coagulase-positive and most avirulent staphylococci are coagulase-negative. However this, it should be noted, is not the same as saying that all coagulase-positive strains are virulent or that all coagulase-negative strains are avirulent. FIGURE 1 attempts to show diagrammatically a situation in which both the latter statements are false. If the areas in FIGURE 1 labeled coagulase-positive, coagulase-negative, and virulent are proportional to the distribution of these types in nature, then most coagulase-positive strains are avirulent and some coagulase-negative strains are virulent. A similar argument may be made for any other property found more often in virulent than in avirulent strains.

If the coagulase test is too coarse a method for identifying virulent strains, might some other property be more useful for this purpose? Of the numerous such properties suggested, we have been most interested in DNase production.

Brown[6] found that β-hemolytic streptococci produced nucleases for RNA and DNA, whereas α-streptococci and enterococci did not. Weckman and Catlin,[7] using a viscometric method, showed that coagulase-positive staphylococci produce significantly greater amounts of DNase than do coagulase-negative strains. Using the agar plate method of Jeffries *et al.*[8] for demonstrating nuclease activity, DiSalvo[9] confirmed the correlation between coagulase and DNase production in staphylococci. TABLE 2 shows the results obtained when we applied this method to the examination of 500 recently isolated strains of staphylococci.

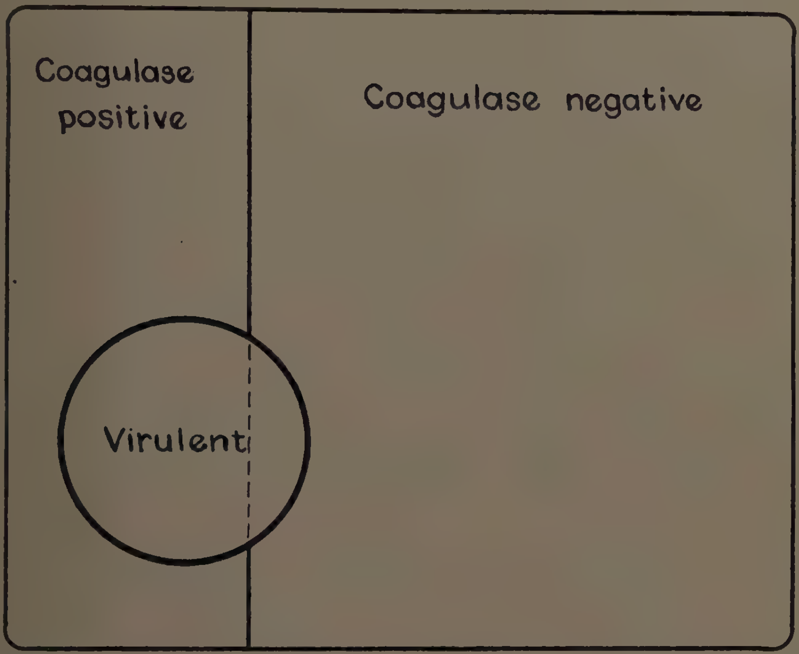

FIGURE 1. Diagrammatic representation of one possible relationship between virulence and coagulase test.

TABLE 2
RESULTS OF COAGULASE AND DNASE TESTS ON 500 STRAINS OF STAPHYLOCOCCI

Source	Coagulase-positive DNase-positive	Coagulase-positive DNase-negative	Coagulase-negative DNase-positive	Coagulase-negative DNase-negative
Suppurative lesions (124 strains)	96	2	4	22
Other clinical sources (169 strains) (including blood, sputum, and urine)	62	7	20	80
Nasal carriers (137 strains)	51	3	4	79
Normal skin and hospital air (70 strains)	5	4	5	56
	214 (42.8%)	16 (3.2%)	33 (6.6%)	237 (47.4%)

From TABLE 2 it can be seen that 96 of 124, or 77 per cent of cultures from suppurative lesions were positive for both coagulase and DNase tests. Only 62 of 169 (37 per cent) of cultures from other sources such as blood, sputum, and urine, were positive for both tests. Similar figures were obtained with cultures from healthy nasal carriers. Only 5 of 70 strains from normal skin and hospital air were positive for both properties. The over-all correlation between coagulase and DNase was 90 per cent. This correlation was higher among strains from suppurative lesions and lower for strains from other sources.

We next compared 200 strains of staphylococci for 6 additional properties that have been used for distinguishing between virulent and avirulent strains. The 6 additional tests employed were: pigmentation on milk agar, gelatin liquefaction on gelatin plates, production of acid from mannitol, the egg yolk opacity reaction of Gillespie and Adler,[10] growth and reduction of tellurite on glycine-tellurite agar,[11] and growth and reduction on agar media containing triphenyltetrazolium chloride.[12]

TABLE 3 shows that of 173 strains that were positive for coagulase and DNase, 127 (73.5 per cent) were also positive for all of the 6 additional properties tested. When we examined more closely the sources of our cultures, it was found that almost all cultures obtained from suppurative lesions fell into the first category; that is, these cultures were almost always positive for all of the tests employed. Most of the cultures that were coagulase-positive and DNase-positive but negative for one or more of the other tests were obtained from sources such as sputum or urine where staphylococcal infection was more open to question.

TABLE 3

COMPARISON OF COAGULASE, DNASE, AND SIX OTHER TESTS ON 200 STRAINS OF STAPHYLOCOCCI

Number of additional tests positive*	Coagulase-positive DNase-positive	Coagulase-positive DNase-negative	Coagulase-negative DNase-positive	Coagulase-negative DNase-negative
6	127	0	1	0
5	26	0	1	0
4	12	0	1	0
3	7	0	0	1
2	1	3	0	7
1	0	2	1	7
0	0	0	0	3
	173	5	4	18

* The additional tests were: pigment, gelatin liqufaction, egg yolk reaction, production of acid from mannitol, growth and reduction on tellurite and tetrazolium agar media.

Without an objective test for virulence, further cultural reactions of this sort can not be expected to lead to any definite conclusions. It is clear that virulent staphylococci are biochemically more active than their avirulent relatives and that they are more consistently positive for a variety of biochemical reactions that might be tested. However, so far we have avoided the basic question: What is a virulent staphylococcus?

The question is not so difficult to answer given a willingness to withhold judgment on some strains and convict only those caught in the act of pathogenesis. A virulent staphylococcus is one that is obtained in pure culture from an accessible and typical lesion, a pyogenic abscess. Such strains are *ipso facto* pathogens.

However, are they fortuitous pathogens, opportunist pathogens, potential pathogens that happened to find the proper conditions for multiplication in a susceptible host, or are they pathogens by virtue of their intrinsically virulent nature? If they are the latter, might this virulence still reveal itself, if not by a single biochemical property, then by some combination of such properties?

With these thoughts in mind, we collected a series of strains from lesions of undisputed staphylococcal etiology and compared them with other strains that, when isolated, were not presently engaged in pathogenesis. Each culture was submitted to 15 *in vitro* tests. These tests were: production of coagulase, fibrinolysin, α- and δ-hemolysins, RNase, DNase, phosphatase, lipase (egg yolk reaction), and pigment; fermentation of mannitol, ribose, and trehalose; gelatin liquefaction, and reduction of potassium tellurite and of triphenyltetrazolium chloride.

TABLE 4

COMPARISON OF 40 STRAINS OF STAPHYLOCOCCI FROM KNOWN SOURCES

Source	Number of strains in group	Number of strains positive for all tests	Number of positive tests in group	Number of strains resistant to pen, SM, & tetracyclines	Number of strains phage type 80/81
Lesions, hospital personnel	10	10	150	10	10/10
Lesions, patients	10	9	149	8	9/10
Nasal carriers	10	4	140	4	1/10
Normal skin	10	0	39	2	—

TABLE 4 summarizes the results of these experiments. The first group listed in the table, those cultures from hospital personnel, were all clearly defined cases involving single or multiple boils in nurses and physicians. Each of the 10 cultures was positive for each of the 15 *in vitro* tests, giving a total of 150 positive tests. All 10 cultures were resistant to penicillin, streptomycin, and tetracyclines, and most of these cultures were resistant to other antibiotics as well. All 10 of these cultures were phage type 80/81.

In the second group of cultures, 5 were obtained from inpatients and 5 from outpatients. One of these strains was obtained in essentially pure culture from the sputum of a patient with a clinical picture consistent with a diagnosis of staphylococcal bronchopneumonia. Another was isolated from the bloodstream of a toxic patient who had had a boil on the neck just prior to hospitalization. The other 8 cultures in this group were from abscesses. A single culture in this group failed to reduce triphenyltetrazolium chloride. This was the only negative test among the 10 strains and the 15 tests. This culture, which was the one isolated from blood, was sensitive to all antibiotics tested and was the only strain in this group which was not phage type 80/81. It was type 29/79/80.

The 10 strains from healthy nasal carriers were not randomly chosen strains, but were rather 10 strains selected because they appeared to be biochemically similar to the abscess strains. All were coagulase-positive, DNase-positive and, when originally isolated and tested, positive for the additional 6 tests listed in TABLE 3. However, when still further tests were applied to this group, only 4 of 10 remained positive for all tests. There were 10 negative tests within the group, only 4 cultures were resistant to penicillin, streptomycin, and tetracyclines, and only one culture was phage type 80/81.

The 10 strains isolated from normal skin were all coagulase-negative and DNase-negative, and were included to show the comparative lack of activity of such strains in the tests employed.

From TABLE 4 it is seen that the virulent staphylococcus is not only biochemically versatile but remarkably consistent in its reactions. Strains isolated from lesions are more likely to be positive for all tests than are strains isolated from healthy hosts. The abscess strains appear to be physiologically homogeneous. They usually belong to relatively few phage types and are often resistant to several, or most, of the commonly used antibiotics. They are recognized frequently enough to have won the sobriquet "epidemic" or "hospital" strain. Epidemiological surveys by others, utilizing phage-typing techniques, indicate that these strains are the ones found most often in pathologic processes, much less frequently in the healthy nose and throat, and only rarely are isolated from normal skin, air, or fomites. The results of our physiological tests are in agreement with this viewpoint.

However, we still lack a single, definitive characteristic for separating the epidemic strains from others that mimic them. It is possible that such a characteristic reveals itself only *in vivo*. We have sought such a characteristic by growing staphylococci under conditions that might resemble in one way or another those found in lesions. These attempts to find a single distinguishing property for virulent staphylococci have been unrewarding so far and will not be discussed here. However, as an example of the sort of reasoning employed, the following might be offered. Staphylococci are usually studied *in vitro* under aerobic conditions. The interior of a walled-off lesion is probably an environment of low oxygen tension. The ability to carry out certain functions anaerobically may therefore be the distinctive mark of a pathogen. TABLE 5 summarizes the information we have at our disposal concerning various properties of virulent and avirulent staphylococci grown aerobically and anaerobically. (Some of this information was taken from the literature and some from our own experiments.)

It can be seen from TABLE 5 that some properties that distinguish virulent from avirulent strains aerobically are not present anaerobically. These are the ability to form pigment, produce α- and δ-hemolysins, split ribonucleic acid, and lyse fibrin. However a perfect biochemical distinction between virulent and avirulent strains still eludes us.

Staphylococcal disease has been discussed here so far in terms of abscess formation, as if localized infections are the only kinds that staphylococci can produce. This of course is not so.

Rammelkamp and Lebovitz[13] have recorded their impression that abscess formation is rare in infants. Certainly the more disseminated forms of staphylococcal disease, such as impetigo and pneumonia, are more common in the pediatric age group than in adults. Just as it is possible to discuss virulence without reference to host factors, it is possible to consider localization and dissemination in the host without reference to virulence in the microbe. The same organism that is capable of producing an abscess by virtue of its intrinsic virulence may spread from its favored subcutaneous sites to other tissues where it may give rise to osteomyelitis, pneumonia, septicemia, and even meningitis.

When isolated from acute, generalized infections, the virulent staphylococcus usually exhibits the same broad spectrum of biochemical activity that has been described for the abscess strains. However in other cases, usually those of a more chronic nature, strains may be recovered that show few of the properties of the abscess strains. How can we account for the unquestionable pathogenicity of these strains? A consideration of variation in staphylococcal cultures may provide an answer.

TABLE 5

SOME PROPERTIES OF VIRULENT AND AVIRULENT STAPHYLOCOCCI CULTURED AEROBICALLY AND ANAEROBICALLY*

Property	Aerobically		Anaerobically	
	Virulent	Avirulent	Virulent	Avirulent
Catalase	+	+	+	+
Coagulase	+	−	+	−
Enterotoxin	+	−	+	−
Tetrazolium reduction	+	−	+	−
Deoxyribonuclease	+	−	+	−
Ribonuclease	+	−	−	−
alpha Hemolysin	+	−	−	−
delta Hemolysin	+	−	−	−
Pigment	+	−	−	−
Gelatin liquefaction	+	−	−	−
Fibrinolysin	+	−	−	−
Acid from mannitol	+	usually −	+	−
Uracil requirement	no	no	yes	yes(?)

* +, = positive; −, = negative.

It is a familiar observation that when a pigmented culture is plated out, some nonpigmented colonies may develop. Elek and Levy[14] have shown that individual clones of a hemolytic culture may vary widely in their ability to produce hemolysins. Meyer[15] picked individual colonies for coagulase testing; as many as 3 of 10 colonies were coagulase-negative when the entire culture was strongly coagulase-positive. Active cultures may therefore harbor inactive variants.

A simple technique demonstrates that the reverse situation is also possible; that is, that inactive cultures may contain a few active variants. Virulent abscess strains that are coagulase-positive and DNase-positive grow luxuriantly on a washed agar medium containing only glucose, casein hydrolysate, thiamin, and niacin. Avirulent strains have additional growth requirements. These additional requirements may sometimes be supplied by biotin alone, but often other factors are also required. If virulent clones occur within predominantly inactive cultures, growth on the minimal medium supplemented only with thiamin and niacin should bring out the active variants.

We have studied a number of coagulase-negative strains by this technique. In one notable instance, a coagulase-negative, DNase-negative, and otherwise biochemically inactive strain was recovered ante mortem on 5 separate occasions from the blood stream. Post mortem, a similar culture was isolated from vegetative lesions on the heart valves. When these cultures were plated onto minimal agar containing only glucose, casein hydrolysate, thiamin, and niacin, a few yellow-pigmented colonies were recovered. Subcultures of these colonies had like characteristics, including coagulase-positive and DNase-positive. Biochemically, these subcultures from minimal agar resembled in every respect the abscess or epidemic strains, whereas the original cultures, maintained on tryptose agar, remained negative for these tests and resembled skin strains. Similar results were obtained with a coagulase-negative strain isolated from an eye.

These experiments require confirmation with a larger number of cultures. They suggest, however, that virulent strains of staphylococci may become stabilized at a high level or at a low level of biochemical activity. Lack[16] has suggested that in chronic lesions nontoxigenic strains may become predominant as a result of the absence of selection pressure on the part of the host. In the active lesion, this selective pressure is probably much greater. An interesting report by Braun and Whallon[17] indicates a mechanism by which a high level of activity may be maintained by staphylococci in active lesions. These authors found that the depolymerization products of DNA favored the emergence of S forms in cultures of *Brucella*. In the staphylococcal abscess there are both DNA (from pus) and DNase (from the staphylococci).

All the tests discussed here were qualitative tests. The abscess or epidemic strains always gave clear-cut, strongly positive reactions under the conditions employed for performing these tests. However, strains giving negative reactions may have actually possessed the property in question but to an extent too small to be detected by these qualitative methods.

If the biochemical activity of a culture represents the sum total of the activities of the individual cells that make up the culture, then a quantitative approach may explain both actual and potential virulence. Actual pathogens may then be those strains stabilized a at high degree of biochemical activity, and potential pathogens those strains that have decreased over-all biochemical activity but that contain within themselves units, cells or clones, of high activity.

The results of Elek's experiments[18] on human volunteers might be explained on this basis. Using as a measure of virulence pus-production following intradermal injection of known numbers of cocci, it was found that doses of the order of one million cocci were required to produce infection. As Elek pointed out, such large numbers of organisms are not likely to be encountered in nature. However, infections do occur in nature, and foreign bodies, which in Elek's experiments lowered the minimum infecting dose, are not always involved. It is possible that in some of the cultures used one million cells were necessary to include a few truly virulent cells. One of Elek's strains was a biochemically active abscess strain and did not produce pus when injected in numbers as high as 2000 cells. However it apparently did produce pus in the patient from whom it was isolated. Many of the strains were nasal strains. It is of interest that one of these nasal strains that did not produce pus when more than a million cells were injected was coagulase-positive and lipase-positive but was white in color, mannitol-negative, fibrinolysin-negative, and negative for α-hemolysin.

In summary, taking the pyogenic abscess as the typical staphylococcal lesion and taking the strains isolated from such lesions as the prototype of the virulent staphylococcus, virulent staphylococci are found to be physiologically active and remarkably consistent in their biochemical reactions. These virulent staphylococci appear to comprise a single strain or a very few closely related strains. Such strains can not be recognized by the use of a single *in vitro* characteristic, such as the ability to clot plasma, for this includes too many strains that are for practical purposes avirulent.

If this more restricted concept of virulence is allowed, consequences of epidemiological and immunological importance follow.

Epidemiologically, we may profit by concentrating attention on a less miscellaneous group of organisms. It would seem that man acquires the virulent staphylococcus, as he does the tubercle bacillus, chiefly from other open cases. Energetic efforts in hospitals to purge the air of micrococci by such devices as replacing the old air-conditioning system, oiling the blankets, and adding another disinfectant to the mop pail might be replaced by more strict attempts to isolate patients and personnel with active staphylococcal infections.

Immunologically, a more narrow definition of virulence might alter our concepts of host resistance. The usual assumption, arrived at from the fact that man lives and thrives in a staphylococcus-laden environment, is that his natural resistance to infection with these organisms is quite high. The evidence for this high resistance disappears if it is accepted that most of the staphylococci in man's environment are neither pathogens nor potential pathogens. Our impression after observing many instances of staphylococci establishing themselves in otherwise healthy hosts is that man has little resistance against the truly virulent strains of these organisms except his ability to localize the infections they produce.

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BIOCHEMICAL PROPERTIES OF VIRULENT AND AVIRULENT STRAINS OF BACTERIA: *SALMONELLA TYPHOSA* AND *PASTEURELLA PESTIS*

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Salmonella typhosa

In nature simple invasion does not result in disease; it is only following multiplication of the invading organism that disease may become apparent. Since growth *in vivo* is essential for the pathogenic behavior of an organism (although not all organisms growing *in vivo* are pathogenic) it would be expected that failure to multiply, for whatever reason, would preclude pathogenic activity. In experimental infections, however, disease can result from administration of a sufficiently large number of organisms without the need for their subsequent multiplication or by injection of killed cells. The relative virulence of organisms is inversely related to the numbers required to produce the defined pathogenic effect following experimental infection. In this article I define a virulent strain of *S. typhosa* as one having an average lethal dose (a.l.d.) of less than 50 million organisms when injected intraperitoneally in buffer into mice and an avirulent strain as one having an a.l.d. in excess of 100 million under the same conditions.

Among the biochemical processes most likely to influence the ability of an organism to grow in a particular environment, those concerned with the synthesis of new bacterial matter are of fundamental importance. Failure of any one essential synthesis in the environment provided by the host would result in nonpathogenicity in nature and avirulence in experimental infections. Means whereby strains incapable of growth *in vivo* could be obtained became evident from studies on the effects of biochemical mutation on the virulence of *S. typhosa*.[1] These studies were initiated to determine whether the general complexity of the nutritional requirements of pathogenic species as a whole contributed to or resulted from their pathogenic nature. Auxotrophic mutants of many different types were derived from a single prototrophic strain. No association between increased nutritional complexity and increased virulence was observed. On the contrary, mutants requiring certain factors for growth had markedly reduced virulence, and this reduction subsequently has proved to be a useful finding. Requirements for exogenously supplied purines or for para-aminobenzoic acid (PABA) were particularly effective in reducing the virulence of auxotrophic strains.[2] Detailed studies with mutants of these two types led to the conclusion that their avirulence resulted from failure to multiply through nonavailability of their specific requirements *in vivo*.[3] The evidence on which this conclusion was based is briefly as follows.

The reduction in virulence was not accompanied by detectable antigenic alterations. Peritoneal fluid derived from normal mice and added to minimal medium did not provide factors utilizable by purine- or PABA-dependent organisms but permitted abundant growth of auxotrophs having other requirements and retaining full virulence. The number of organisms recoverable from mice moribund from infection with lethal doses of purine- or PABA-dependent organisms was always smaller than the number injected. From mixed infections with large numbers of purine- or PABA-dependent organisms and small numbers of prototrophic parental cells, the *in vivo* population at death consisted almost exclusively of parental organisms. Mice injected with nontoxic amounts of purines or PABA permitted auxotrophs with these requirements to show high virulence and to achieve high population levels *in vivo*. Purines specifically enhanced the virulence of purine-dependent but not PABA-dependent organisms; PABA enhanced PABA- but not purine-dependent organisms. Whereas it was necessary to inject purines parenterally to obtain virulence enhancement, PABA was effective when added to the diet. On reversion to the prototrophic condition, both types of mutant became fully virulent for normal mice, again without detectable antigenic alterations.

The fundamental biochemical differences between avirulent purine- or PABA-dependent organisms and fully virulent prototrophic strains can be deduced with some certainty. Abundant evidence exists from studies in bacteria and molds that auxotrophy most frequently results from inhibition of one or more steps in biosynthesis, through gene mutation with consequent failure to produce an enzyme essential for the synthesis of the required factor. In all probability purine- and PABA-dependent strains differ from a fully virulent strain in single gene functions. With the recent discovery of the operation of genetic transfer systems in *S. typhosa*,[4] it may now be possible to confirm this directly. Studies on avirulent, purine-dependent strains of *S. typhimurium* showed that genetic transduction with phage could convert them to purine-independent strains with concurrent restoration of virulence for mice.[5]

Although the *Salmonellae* have received the extensive study their medical and bacteriological importance merits, surprisingly little is known about the determinants of virulence and pathogenicity in this genus. Strains and species differ widely in their host specificities and in the forms of disease they produce in susceptible hosts. There can be little doubt that these differences have biochemical bases and depend on factors additional to Vi antigen and the toxic O somatic complex.

Pasteurella pestis

The causative agent of plague in rodents and in man, unlike *S. typhosa*, has high virulence for normal laboratory animals and is an appropriate organism for the experimental study of this important property of pathogenic organisms. With reference to *P. pestis* I will define virulent strains as those having a.l.d. for mice and for guinea pigs of less than 20 organisms injected in buffer either subcutaneously or intraperitoneally. Strains having a.l.d. in excess of this figure will be referred to as avirulent. The term avirulent will thus include strains of reduced virulence (a.l.d. 10^2 to 10^7 organisms) together with frankly avirulent strains (a.l.d. for mice exceeding 10^8, for guinea pigs exceeding 10^{10} organisms).

Apart from changes in virulence attributable to nutritional deficiences referred to below, the biochemistry of virulence in *P. pestis* is poorly understood. This stems in large part from lack of knowledge, until recently, of the properties (other than virulence itself) that differentiate virulent from avirulent strains of this pathogen. Much confusion must have resulted in the past from lack of appreciation that avirulence can result from loss of any one or more of a number of virulence-determining characters to give avirulent strains of many different types (TABLE 1). Differences detected between a virulent strain and an avirulent representative would be confirmed only with avirulent strains of the same type and refuted by avirulent strains of other types.

The virulence determinants at present recognized can be conveniently symbolized F1+, VW+, P+, and Pu+, and only strains possessing all are virulent both for mice and for guinea pigs. It has not yet been possible to assess whether toxigenicity (T+) is an important determinant but I would be surprised if it were not. Knowledge of the above determinants and of properties associated with them forms the basis on which biochemical studies may be planned. I shall briefly review this knowledge, taking each determinant separately, and try to avoid laboring our ignorance of the biochemistry underlying their manifestation.

TABLE 1

SOME REPRESENTATIVE AVIRULENT STRAINS DEFICIENT IN ONE OR MORE VIRULENCE DETERMINANTS

One deficiency*			Two deficiencies			Three deficiencies
F1−	VW−	P−	F1− VW−	F1− P−	VW− P−	F1− VW− P−
M17	M15	M14	M16	M18	M32	M33
M23	A1122	EV 76	14	M19	Java	TRU
	Tjiwidej	Harbin		M25	017	
	Soemedang	Elizabeth			020	
		Butembo				
		M7				

* Strains are "+" for all other virulence determinants; for example, M14 is F1+ VW+ P− Pu+.

Pu+. As anticipated from studies with *S. typhosa*, mutation of a purine-independent (Pu+) virulent strain of *P. pestis* to purine-dependence (Pu−) resulted in avirulence without detectable alteration in other properties. The effect in terms of the a.l.d. was more dramatic than had been observed with *S. typhosa*; mutation from Pu+ to Pu− raised the a.l.d. for mice by a factor of 10 million and for guinea pigs by one of 1000 million. The reason for this great reduction in virulence and its biochemical basis are presumed to be as concluded for analogous mutation in the typhoid organism. We have found Pu− strains to be useful tools in the study of immunity to experimental plague.[6]

F1+. It was an early finding in the study of *P. pestis* that *in vivo* and, under appropriate conditions *in vitro*, the organisms were surrounded by a capsule or envelope.[7] Whether the envelope of organisms grown *in vitro* is identical with that produced *in vivo* has not been proved. Material thought to comprise the envelope of organisms grown *in vitro* was extensively studied by Meyer and his associates and considered to be a protein.[8] It is antigenic and most commonly referred to as fraction 1, although other names have been used.[9,10,11] Strains competent to produce fraction 1 (F1) as an envelope surrounding the organisms, under appropriate conditions, are symbolized as F1+; those incompetent to produce this material under any conditions are F1−.

F1 antigen accumulates as a visible envelope when F1+ organisms are cultured at or near 37° C.; at the optimum temperature for growth, 28° C., negligible surface accumulation of F1 occurs. This antigen nevertheless is present in or on organisms grown at 28° C., from which it may be liberated by ultrasonic treatment[16] and detected by haemagglutination or agar diffusion methods. F1− organisms can be selected easily from F1+ populations with the aid of F1-specific antisera.[6] Such organisms do not accumulate a surface envelope, are not agglutinated by F1 antisera, do not liberate F1 antigen after ultrasonic treatment, and do not give detectable F1 antibody when used as vaccines. They appear to be genetically incompetent to synthesize F1 antigen. As F1 was considered for many years to be of greatest importance in determining virulence in this organism, it was an unexpected finding that strains rendered F1− retained virulence for mice. They were, however, avirulent for the guinea pig (a.l.d. 10^3 to 10^6 organisms). F1 antibody did not protect mice against infection with F1− virulent organisms, in contrast to its efficiency in protecting against F1+ infection.[12]

In addition to frankly F1+ and F1− types a third occurs having some properties of the former and some of the latter. Strains of this third type do not accumulate F1 antigen as an envelope at 37° C. or at 28° C. nor when growing *in vivo*. They do, however, synthesize this antigen, as can be shown by agar-diffusion methods and by their ability to elicit F1 antibody. In virulence titrations they behave as F1−, and F1 antibody does not protect against them. In immunogenicity studies they behave as F1+. I suggest that they be symbolized as F1±. Two strains of this type have been isolated in our laboratory and a third recovered from a human plague fatality (strain Bryan).[6]

The accumulation of F1 by F1+ strains cultured at 37° C. but not at 28° C. could result either from increased production or less efficient utilization at the higher temperature. There are many precedents for the second alternative, for example the accumulation of intermediate metabolites following mutational blockage of enzyme syntheses. *P. pestis*, at least *in vitro*, is more perfectly organised for the biosynthesis of essential bacterial matter at 28° C. than at 37° C. and has greater synthetic ability, as evidenced by its simpler nutritional requirements, at the lower temperature.[13-15] In the absence of more precise information, which may eventually be obtained by studies such as those of Fox and Higuchi[16] or by a genetic approach, it would seem probable that F1 antigen accumulates on F1+ organisms as a result of blockage of its further utilization at 37° C. If so, we must presume that in F1± organisms further metabolism of F1 can proceed at the higher temperature. In some exploratory experiments, F1+ organisms were grown at 28° C. and converted into protoplasts by incubation with penicillin and sucrose at this temperature. Diffusible F1 antigen could not be detected in such protoplasts following their rupture by osmotic shock. However, such ruptured protoplasts efficiently removed F1 antibody from antisera, suggesting the existence therein of a nondiffusible, and therefore presumably complexed, material having F1 specificity. Prior incubation of protoplasts for 6 hours at 37° C. before rupturing reduced the amount of nondiffusible F1-containing material by 75 per cent.

F1− organisms either are genetically incapable of producing F1 antigen or they convert it into other products as rapidly as it is formed. It has not been possible to identify an alternative antigen produced by F1− organisms but not by F1+, suggesting that if other products are formed they are not antigenic.

VW+. All virulent strains examined can produce, under appropriate conditions, two antigens that I have called V and W. They are therefore symbolized VW+. Strains failing to produce these antigens (VW−) are universally avirulent.[17] As with the other determinants, possession of the ability to produce V and W antigens does not in itself confer virulence. VW+ organisms, whether virulent or avirulent, have the ability to develop resistance to phagocytosis by mouse polymorphonuclear cells in the absence of visible envelopes. The possibility that such resistance was mediated by an invisible but effective coating of F1 antigen (which too can give resistance to phagocytosis) was refuted when F1− and VW+ organisms became available for study. It has recently been reported that VW+ organisms resistant to phagocytosis by polymorphonuclear cells are readily ingested by monocytes, in which they multiply and later are released from them.[18]

V and W antigens are most easily demonstrated and assayed by the agar-diffusion technique. Like F1 antigen, they accumulate maximally at 37° F. rather than at the optimum temperature for growth. Their destruction by trypsin and by Sevag treatment and their resistance to periodate suggest they are proteins. Centrifuged broth cultures of VW+ organisms grown under conditions suitable for V and W production show most of the W antigen to be contained in the supernatant. Of the V antigen about one half is in the supernatant and one half deposited with the organisms. Both antigens can be precipitated from culture supernatants by half-saturation with ammonium sulfate, but separation of one from the other and from other antigens by fractional precipitation has not shown great promise. Material relatively enriched in W or V can be obtained respectively in precipitates formed by 30 to 35 and 35 to 40 per cent saturation with ammonium sulfate. Neither antigen has as yet been obtained sufficiently pure and in sufficient quantity for chemical study. While crude antigens are stable in solution in the cold (2° to 4° C.) and are nondialyzable, more purified preparations appear first to double their apparent concentrations, then each to give double lines of precipitation in agar diffusion plates and, finally, to become undetectable by diffusion methods on prolonged storage. Solutions of purified products became serologically inactive on dialysis against phosphate buffer *p*H 7: activity could not be found either in the diffusate or the dialysate or in the two combined. The isolated products would thus appear to be unstable and dissociate into smaller units that eventually are not effective precipitins. Strains that produce V but not W antigen or, vice versa, have not been obtained, suggesting that these antigens have a common precursor or that they are themselves dissociation products of a larger entity.

In addition to the obvious difference between VW+ and VW− strains relating to specific antigen synthesis, other associated properties are now recognizable. Higuchi and Smith[19] reported that virulent organisms were unable to grow on agar at 37° C. in the absence of available calcium, whereas the growth of avirulent organisms was not impeded. This observation has been confirmed to the extent that plating on Ca-deficient medium selectively suppresses VW+ organisms and not VW− rather than permitting only growth of avirulent strains. Neither type is inhibited on this medium at 28° C. The use of calcium-deficient medium permits easy selection of VW− strains from VW+ populations.

VW+ organisms have lower growth rates than their VW− derivatives in broth cultures growing at 37° C. with aeration, which results in rapid enrichment of initially VW+ cultures with VW− organisms and corresponding reduction in the virulence of the whole cultures.[20] Unlike the situation with F1− strains, VW− strains differ from VW+ in having larger amounts of another antigen—antigen 4 in the scheme of Crumpton and Davies.[21] This distinction is probably a qualitative difference between the two types, the small amount of antigen 4 detectable in VW+ cultures arising from the unavoidable admixture of such cultures with a proportion of spontaneously arising VW− organisms. Like F1 and VW, antigen 4 accumulates maximally at 37° C. Assuming that strains can possess either antigen 4 or V and W antigens but not all, simple schemes could be visualized to accord with the situation. Antigen 4 could be a precursor of V + W, in which case VW− strains could be blocked in the further metabolism of antigen 4, leading to its accumulation or, alternatively, V + W could be the precursor of antigen 4, in which case VW+ strains would show the inhibited synthesis. Under the first scheme (4 → VW) it theoretically should be simple, by loss of biosynthetic activity, to derive VW− strains from VW+. As stated above, such derivatives arise very frequently. The alternative scheme (VW → 4) should permit the production of VW+ strains from VW− by loss of synthetic ability. This occurrence is extremely rare and has never been conclusively demonstrated. It would be interesting to attempt to demonstrate conversion of antigen 4 to VW or vice versa, using cell-free extracts.

P+. In a comparative study of the nutritional requirements of a virulent and an avirulent strain (now known to be avirulent through being VW−) both strains were found to require the addition of glycine, cystine, methionine, and phenylalanine for growth on glucose-ammonia-phosphate agar at 28° C. Hemin or bisulfite addition was necessary for the development of isolated organisms. On hemin-supplemented media both types produced brown-pigmented colonies (P+) from which nonpigmented (P−) secondary colonies arose on continued incubation (formerly the two types were called P and NP, respectively). P− colonies, on replating on the above medium, gave pure P− populations, and "reversion" from P− to P+ was never observed. Interest was attracted by the great reduction in virulence resulting from mutation of a virulent P+ strain to P−. The generality of this observation was confirmed with a number of different virulent strains. Some strains gave derivatives showing degrees of pigmentation intermediate between P+ and P− types. Pigmentation of colonies resulted from absorption of hemin from the medium.[22]

P− strains derived from virulent parents (that is, F1+, VW+, P−, and Pu+ strains), although avirulent for normal animals, showed full virulence in mice previously injected with small nontoxic doses of iron salts. This was a specific effect of iron, other metals in similar or larger doses giving no enhancement. Doses of 2.5 μg Fe (as ferrous sulfate in phosphate buffer) gave detectable enhancement of virulence either when injected with the challenge organisms or when injected 7 days before challenge. The virulence-enhancing effect of iron so easily demonstrated in mice was less evident in guinea pigs. In these animals, increasing doses of injected iron reduced the a.l.d. progressively from approximately 10^{10} to 10^4, but higher amounts of iron, which by extrapolation would have been expected to reduce the a.l.d. to nearly 10 organisms, were themselves toxic and could not be used. Iron at nontoxic levels did not materially enhance the virulence of P− strains derived from VW− avirulent parents. Thus, provided strains had all other virulence determinants, the impediment to high virulence resulting from being P− could be removed by the provision of iron *in vivo.*

When tested in normal mice the a.l.d. of a P− derivative (M7) of a fully virulent P+ strain (M3) was poorly defined over the range 10^3 to 10^7 organisms, there being no consistent relationship between the injected dose and resulting response. The population of organisms in animals moribund from doses over this range was low and never in excess of 10^8 organisms per mouse, in contrast to populations of 10^{10} achieved by P+ strains. In the presence of injected iron (20 μg Fe) P− organisms resembled P+ in producing high populations and in having an a.l.d. of approximately 10 organisms. These observations were interpreted to indicate that normal mice (Porton strain) had low but variable levels of iron available to P− organisms. In some, but not in others, the level was sufficient to permit the development of lethal populations; the level of available iron, rather than the dose of organisms, decided the outcome of infection. Provision of additional iron permitted lethal populations to develop in all challenged animals.[23]

The reported behavior of smooth (S) and nonsmooth (NS) strains isolated from cultures by Eisler[24] suggested their correspondence with our P− and P+ types, respectively. The important difference between S and P− types, however, was that some S strains retained high virulence for normal mice. Through the courtesy of Eisler these strains were made available for tests in our animals. Here they were P− and avirulent, suggesting that different mouse stocks may differ in available iron content. It has not been possible to demonstrate that added iron at virulence-enhancing levels interferes with phagocytosis by polymorphonuclear cells *in vitro* or *in vivo*, nor does it enhance the toxicity of killed organisms. These observations, together with the fact that iron does not enhance VW− organisms, support the conclusion that P− organisms are population-limited *in vivo* by iron deficiency. It is quite evident that P+ organisms are not limited in this way. However since injected iron has been found consistently to reduce the a.l.d. of virulent strains to one organism and to reduce the mean time to death without reduction in terminal population levels, it is probable that iron metabolism is a growth-rate limiting factor in the development of P+ strains *in vivo.*

The biochemical problem with respect to P+ and P− would seem to resolve into an identification of the mechanism whereby P+ strains are capable of deriving a sufficiency of iron *in vivo*. The hypothesis that they could derive iron from a serum-iron complex that P− strains could not utilize was not supported by experiment. Jackson (unpublished data) has shown that both P+ and P− types are iron-limited in freshly and carefully withdrawn mouse, guinea pig, and human sera. Their quantitative responses to added iron in such sera did not differ. It has not been possible to show conclusively *in vitro* that there are antigenic differences between P+ and P− strains. There are suggestions that an antigen of P− exists in the haptenic form in P+, as judged by diffusion studies with antisera produced in guinea pigs. Comparative immunogenicity studies in mice and guinea pigs indicate that an antigenic difference between P+ and P− does exist, as also do Eisler's studies with S and NS types.[25]

It is not at all certain that the mechanisms permitting pigmentation on defined media are themselves important in determining the behavior of P+ organisms *in vivo* or whether this behavior is determined by a property associated with the ability to pigment. This doubt has arisen from some observations on the closely related organism *P. pseudotuberculosis* that, like *P. pestis*, can give rise to VW+ P+ and VW+ P− types.[26] Both P+ and P− here are avirulent for normal mice and both are virulent for mice injected with iron. Thus, although P+ types can form pigmented colonies, they still require injected iron to permit the development of lethal populations *in vivo*, whereas P+ strains of *P. pestis* do not have such a requirement. The two species resemble each other in that iron has no virulence-enhancing action on VW− derivatives.

In summary then, although biological differences between virulent and avirulent strains can be defined with ease *in vitro*, the biochemistry underlying these differences is only superficially understood. Biochemical studies clearly should take the form of detailed comparisons of virulent strains with avirulent strains lacking each known virulence determinant in turn and not with a general collection of avirulent strains comprising many different types. Ideally all strains should have the greatest possible genetic identity. We have selected from a single virulent strain a series of derivative strains showing all permutations of the four virulence determinants F1+, VW+, P+, and Pu+: a collection of 16 strains in all. Of these only one shows maximum virulence both for mice and for guinea pigs, two are fully virulent for mice, and the others have reduced virulence or are avirulent for both species. This collection would provide material on which biochemical studies could most profitably be initiated. It is unfortunate that genetic transfer systems, so useful in the analysis of other genera, have not been demonstrable in the *Pasteurellae*, thus excluding examination of the known virulence determinants at the genetic level. This genus's close taxonomic relationship with members of the *Enterobacteriaceae* showing such systems offers hope that genetic studies with *P. pestis* will eventually become possible and lead in turn to a better understanding of the biochemistry involved in the expression of virulence.

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Discussion of the Paper

Robert P. Williams (*Baylor University College of Medicine, Houston, Texas*): Investigators from Porton, England and from Fort Detrick, Md. have reported that cultures of *Bacillus anthracis* sometimes produce a pink or red pigment. K.-C. Chao in our laboratory has carried out several studies on anthrax pigment production. He has defined the optimum conditions for production and compared the pigmentation of virulent and avirulent organisms. Both virulent and avirulent strains of anthrax produce pigment. Originally we believed this to be the end of the story, but further investigations established that a difference did exist between the two organisms. If the amount of pigment produced per unit cell mass, or pigmentation index, was determined, we observed that virulent organisms always showed a higher value than the avirulent. The data for several pairs of virulent and avirulent strains are presented in TABLE 1. The avirulent strains were isolated from virulent parents by cultivating the latter on bicarbonate-nutrient agar and selecting rough colonies (avirulent) from the smooth (virulent). Mouse inoculations established that virulent strains were lethal at concentrations of 10^2 cells. Avirulent strains would also kill mice if injected in concentrations of 10^6 cells or greater, but the pathology was not that of anthrax.

Of particular interest with respect to the *P. pestis* data as presented by Burrows is the fact that the quantitative difference between pigment production by virulent and avirulent anthrax organisms appears to be related to their iron metabolism. The optimum iron concentration (as $FeSO_4 \cdot 7H_2O$) required for maximum pigment production by the two strains of *B. anthracis* M 36 was determined. The results are shown in TABLE 2 where it can be seen that the iron concentration needed for maximum pigment production was 10 times greater for the avirulent than the virulent strain. However, even at optimum

TABLE 1

PIGMENT PRODUCTION BY VIRULENT AND AVIRULENT STRAINS OF *BACILLUS ANTHRACIS*

Strains[*]	Pigment produced (D_{490})	Cell density[†] (D_{650})	Pigmentation index ($D_{490}/D_{650} \times 10$)
Ba 1 (27)S	0.144	0.619	2.32
R	0.129	0.913	1.41
Ba 1 (36)S	0.128	0.650	1.97
R	0.089	0.852	1.04
Ba 1 (42)S	0.125	0.510	2.45
R	0.065	0.772	0.84
Ba 1 (43)S	0.077	0.845	0.91
R	0.073	0.812	0.90
Ba 1 (44)S	0.068	0.395	1.72
R	0.071	0.793	0.90
Ba 1 (45)S	0.044	0.366	1.62
R	0.062	0.830	0.75
Ba 1 (46)S	0.128	0.680	1.88
R	0.069	0.929	0.74
Ba 1 (48)S	0.101	0.771	1.31
R	0.042	0.585	0.72
Ba 1 (50)S	0.102	0.727	1.40
R	0.062	0.678	0.91
Ba 1 (52)S	0.134	0.748	1.89
R	0.045	0.820	0.55

[*] "S", virulent strain; "R", avirulent strain.

[†] Determined on spectrophotometer after first removing the bacterial capsule by autoclave treatment.

TABLE 2

EFFECT OF IRON CONCENTRATION UPON PIGMENT PRODUCTION BY VIRULENT AND AVIRULENT BA 1 (36)

Iron concentration (μM $FeSO_4 \cdot 7H_2O$)	Virulent (Ba 1 [36]S)				Avirulent (Ba 1 [36]R)			
	D_{490}	D_{650}	PI[*]	Excess iron[†]	D_{490}	D_{650}	PI[*]	Excess iron[†]
None	0.002	0.640	0.03	0.110	0.005	0.753	0.07	0.105
0.0005	0.001	0.672	0.02	—	0.000	0.735	0.00	—
0.005	0.005	0.660	0.08	—	0.000	0.765	0.00	—
0.05	0.123	0.580	2.12	0.148	0.082	0.775	1.06	0.147
0.5	0.120	0.600	2.00	—	0.120	0.713	1.68	—
1.0	0.105	[‡]	—	—	0.118	[‡]	—	—
5.0	0.025	[‡]	—	—	0.030	[‡]	—	—

[*] Pigmentation index.

[†] Before measuring 0.2 mg./ml. of $FeSO_4 \cdot 7H_2O$ was added to the blank and the sample.

[‡] Cell density could not be measured in these cultures because of the precipitate formed by ferrous sulfate.

iron concentration the pigmentation index of the virulent organism was greater. We also demonstrated that when excess iron is added to nonpigmented culture filtrates obtained from virulent or avirulent organisms grown in low iron medium, the culture filtrate became pigmented and the amount of pigment formed was quantitatively the same for both organisms. If the same experiment was repeated with organisms grown in high iron medium, in which case pigment formation occurs but the pigmentation index of the virulent organism is greater, we discovered that addition of excess iron caused the formation of additional pigment in both virulent and avirulent filtrates. Again the total amount of pigment formed was the same for the two strains. The data from these experiments are also shown in TABLE 2.

We believe these results indicate that both virulent and avirulent organisms excrete a substance that, when complexed with iron, forms the pigment. Apparently avirulent organisms utilize more iron for cellular processes than virulent. Therefore, although the bacterial portion of the pigment material is present in the filtrate, there is not enough iron in the medium to combine with the substance to form pigment. The effect is specific for ferrous iron; ferric iron and other metal ions will not form pigment.

In contrast to the pigment of *P. pestis*, the anthrax pigment is not cellular but extracellular. The anthrax pigment is not a heminlike compound but appears to have the characteristics of a peptide. When measured in alkaline solution, the pigment has a broad absorption curve with the peak at 490 mμ. Unfortunately, we have not yet attempted *in vivo* experiments. We do not know whether the simultaneous injection of iron and an avirulent anthrax organism would result in enhanced virulence.

PROPERTIES OF VIRULENT AND AVIRULENT STRAINS OF *PASTEURELLA PESTIS*

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The causative organism of plague has been studied intensively since its discovery in 1894. Summaries of the findings of numerous investigations on the nutrition and physiology as well as other aspects of this organism can be found in recent reviews by Meyer,[1] Girard,[2] and Pollitzer.[3] The available information on biochemical properties of the plague bacillus provides a very useful background for comparative study of virulent and avirulent strains. However, current knowledge does not permit an explanation of pathogenicity in biochemical terms. Most of the properties that have been associated with virulent strains are measured in gross terms far removed from a precise biochemical analysis. This presentation is an attempt to summarize briefly what is known concerning possible virulence factors and to define areas of study likely to increase our understanding of the basis of virulence of this pathogen. The presentation by Fukui, in this monograph, deals with certain aspects of temperature effects on phenotypic expression of virulence.

Fully virulent strains of *Pasteurella pestis* are considered by Burrows[4] to be: (1) toxigenic, (2) encapsulated, (3) able to produce VW antigens, (4) able to develop resistance to phagocytosis in the absence of visible capsulation, (5) able to synthesize purines, and (6) able to produce pigmented colonies on hemin agar. More recently, virulent strains have been reported to differ from avirulent strains by: (1) showing a lethal response in the presence of glucose under certain conditions,[5] (2) having a nutritional deficiency[6] that is satisfied by calcium,[7] and (3) being able to grow within phagocytes.[8,9] Each of these properties will require further study to define biochemically its role in virulence. The properties either required for full virulence or associated with virulent strains are listed in TABLE 1, and are discussed in greater detail in the following paragraphs.

Plague toxin appears to be essential for virulence. Virulent strains and most avirulent strains produce an "endotoxic" protein that is very toxic for mice and rats, less so for monkeys and rabbits, and almost nontoxic for guinea pigs.[1,10] A correlation has been reported by Packer *et al.*[11] between the toxicity of the toxin molecule and its ability to inhibit mitochondrial respiration. Some of the observations on plague toxin that require further study are: (1) the dissociation of the antibody-toxin complex when injected into mice,[1] (2) a greatly increasing differential toxicity for the mouse and guinea pig as the toxin is purified,[10,12,13] and (3) an increase (up to nine-fold) in mouse toxicity of culture lysates[14] or of the soluble portion of cell sonates (unpublished observations) following treatment with sodium desoxycholate or (up to two- to three-fold) following simple dialysis against cold distilled water (unpublished observations).

The VW antigens[15] and the capsule, which is made up mainly of a protein or protein-carbohydrate complex,[16,17] are believed to protect the bacterium from phagocytosis.[18-22] The capsule seems to be required for full virulence;[1,23] however, mutant noncapsulated strains that still produce the VW antigens retain a considerable degree of virulence.[4] Mutant strains lacking the VW antigens are completely avirulent. The VW antigens are produced also by virulent *P. pseudotuberculosis*; of 17 antigens that can be distinguished in *P. pestis*, only 3 have not been found in *P. pseudotuberculosis*.[24,25] The viability and high resistance to phagocytosis possessed by virulent cells grown in the peritoneal cavity of the guinea pig were found to be retained following conversion to protoplastlike forms by treatment with egg-white lysozyme.[26] Cells grown *in vitro* at 37° or 26° C. were relatively insensitive to similar lysozyme treatment.

In addition to producing toxin, capsule (F-I), and "virulence" antigens, fully virulent strains form densely pigmented colonies on defined media containing hemin.[27,4] Nonpigmented strains that retain the ability to make VW antigens demonstrate reduced virulence for mice and a great loss of virulence for guinea pigs; simultaneous injection of iron salts was found to restore full virulence for mice but not for guinea pigs.[28] There seems to be a correlation between the ability of a strain to produce pigmented colonies and the production of a substance in aerated broth at 26° C. that causes cells to adhere to the sides of the flask (unpublished observations). This substance is produced in Difco heart infusion broth containing xylose but not glucose, and its formation is favored by Mg^{++}.

TABLE 1

SOME BACTERIAL PROPERTIES ASSOCIATED WITH VIRULENCE AND FACTORS INFLUENCING THEIR EXPRESSION

Property	Influencing factors
1. Toxin	
2. Capsule } Resistance to phagocytosis	Temperature dependent
3. VW antigens } Resistance to phagocytosis	Temperature dependent
4. Pigmentation on hemin agar	
5. Purine synthesis	
6. Nutritional requirement for Ca^{++}, Sr^{++} or Zn^{++} at 37° C.	Temperature dependent
7. Glucose sensitivity at 37° C.	(a) Temperature dependent (b) Relieved by Ca^{++}, Mg^{++}, Mn^{++} (c) Relieved by fumarate, malate, succinate, α-ketoglutarate
8. Survival and growth in macrophages	Temperature and growth environment, property best developed by growth *in vivo*

Purine-dependent mutants of virulent *P. pestis* were found by Burrows[19] to be avirulent because of the limited availability of purines in the host. Such strains were fully virulent if the purine was injected into the mice simultaneously with the plague bacilli, as was the case in previous work with *Salmonella typhi*.[19]

A temperature-dependent nutritional requirement for Ca^{++} has been associated with virulent organisms. Virulent strains, grown at 26° C. were found to initiate growth very slowly when inoculated into certain broth media and aerated at 37° C.[6,29] Avirulent mutants present in the inoculum or arising in the culture grew without delay and soon predominated the culture. This suppression of growth of virulent cells could be eliminated by lowering the temperature[6] or by adding Ca^{++}, Sr^{++}, or Zn^{++}.[7] Addition of oxalate and Mg^{++} to Difco heart infusion agar has provided a differential plating medium suitable for growth of avirulent cells, while suppressing formation of colonies by virulent cells.[30] The selective growth advantage of avirulent mutants due to their ability to initiate growth immediately in heart infusion broth cultures is nullified by high *p*H, certain culture filtrates, or CO_2.[31,32] All of the virulent strains tested behaved in similar fashion in regard to displacement by avirulent mutants in broth cultures,[6,31,32] and in containing about 0.1 to 0.01 per cent of avirulent cells in stock cultures.[30] A fluctuation test carried out by Higuchi and Smith (unpublished data) indicated a mutation rate of about 10^{-3} from virulence to avirulence in the Alexander strain. The reason for the failure of initiation of growth of virulent cells at 37° C. in the absence of Ca^{++} is not known. The very interesting observation has been made that the addition of Ca^{++}, while stimulating the growth of virulent cells at 37° C., seems to depress V antigen production.[33] It will be of interest to see whether similar effects of calcium can be related to the selective pressure against VW+ *P. pseudotuberculosis* at 37° C. reported recently by Burrows.[24]

A lethal effect of glucose on *P. pestis* cells under certain conditions in aerated cultures has been observed with virulent but not avirulent strains.[5] This glucose effect, observed also with mannitol, is temperature-dependent, occurring at 37° C. or above but not at 36° C. or below. It is relieved by the addition of either Mg^{++}, Mn^{++},[5] Ca^{++}, fumarate, malate, or succinate (G. E. Wessman, unpublished data), and probably also by α-ketoglutarate.[34] Glucose and mannitol were also found by Cohn and Horibata to be the most potent inhibitors of an induced enzyme synthesis (the β-galactosidase system) in *Escherichia coli*.[35] Since glucose is one of the most characteristic and potent of the inhibitors of induced synthesis of many different enzymes,[36] this general mechanism is worth investigating as a possible cause of the lethal effect of glucose on virulent *P. pestis*. Possibly a heat-labile enzyme (such as that shown by Bird and Gots[37] to impose a temperature-sensitive requirement for methionine on a strain of *E. coli*) is inactive at 37° C., while the synthesis of a second enzyme, essential for an alternate reaction, is inhibited by glucose only in virulent cells. Numerous other speculative combinations bear consideration as a basis for further experimentation. Divalent cations may serve to stabilize the heat-labile enzyme. The known effects of metals on proteins include stabilization against heat, other reversible and irreversible denaturation, and proteolytic digestion including self-digestion, as well as activation or inhibition of enzymes.[38] The effects of Krebs-cycle dicarboxylic acids might be to bypass a block in the ability to fix CO_2 for the extra Krebs-cycle synthesis of C_4 dicarboxylic acids as reported in the C-mutants of *Salmonella typhimurium* by Englesberg.[39] Alternatively, the Krebs-cycle dicarboxylic acids might spark the entrance of pyruvate into the Krebs cycle through citric acid formation, a phenomenon that has been associated with virulent strains of *P. tularensis*.[40] Since Wessman (unpublished observations) has some indication that pyruvate also produces the "glucose effect," its possible position in the pathway of metabolism to the active repressor hypothesized by Cohn and Horibata[35] could account for the difference between apparently noninhibitory and inhibitory sugars on a quantitative basis. It may be mentioned in passing that the growth curves of *P. pestis* obtained by Wessman are remarkably similar to growth curves of *B. megaterium* producing megacin.[41]

Virulent *P. pestis* can probably survive and grow within macrophages. Virulent cells of the phagocytosis-resistant or phagocytosis-sensitive type were found to be removed from the circulatory system of the guinea pig at the same rate and to the same extent.[9] This removal was accomplished chiefly in the liver and spleen, presumably by the reticuloendothelial system (RES). Histological evidence (W. A. Janssen, unpublished data) indicated that the phagocytosis-resistant organisms injected intratracheally[42] or intracardially[9] were ingested by the fixed macrophages of the RES and were able to multiply in these cells. Cavanaugh and Randall[8] have recently reported that virulent *P. pestis*, obtained from blocked fleas maintained at 26° C., were susceptible to ingestion by free phagocytes but were able to survive and multiply within the monocytes. The resistance to phagocytosis, which is associated with the synthesis of the capsule (Fraction I) and the VW antigens in virulent *P. pestis* at 37° C., apparently does not prevent their ingestion by fixed macrophages of the RES; it may be, however, that the temperature-dependent "virulence antigens" permit the bacteria to survive and multiply within macrophages.

Table 2

Effect of Capsulation on Infectious and Lethal Response of Guinea Pigs Infected Intradermally

Strain	ID_{50}	LD_{50}
MP6 capsulated	1.2 (0.6–2.5)*	1.2 (0.6–2.5)
M23 noncapsulated	10 (4.8–21)	4500 (1667–12,150)

* Fifty per cent response and 95 per cent confidence limits calculated by the method of Litchfield and Wilcoxon.

Some additional data bearing on the role of Fraction I in virulence have been obtained. The noncapsulated mutant M23, isolated by Burrows[4] from strain MP6, was reported to be fully virulent for the mouse but to show reduced virulence for the guinea pig (intraperitoneal A.L.D., 10^3 to 10^5 for the guinea pig). These findings have been confirmed in our laboratory, and, in the guinea pig infected by the intradermal route, it appeared that the capsule was much more essential for a lethal effect than for infectivity (table 2) (Fukui, unpublished data). Although the number of noncapsulated cells required to establish infection (as indicated by skin lesions, bubo, and temperature rise) was only 10 cells, most of the infected animals survived doses up to 4500 cells. In view of these results and those to be discussed next, further study of the immunoparalysis effects of capsular material reported by Spivak *et al.*[43] is needed to determine its relationship to virulence.

Vaccination of mice with Fraction I (which gives good protection against typical virulent strains) was found by Burrows[4] to give no protection against a noncapsulated mutant that produced no Fraction I. In the process of confirming this observation in the guinea pig, Lawton[33] found that vaccinated guinea pigs were not only unprotected against the noncapsulated strain but appeared to be even more susceptible than controls (TABLE 3). Guinea pigs immunized with microgram quantities of Fraction I (to avoid the immunoparalysis caused by milligram amounts)[43] in oil adjuvant were solidly immune to the capsulated strain but seemed to be more susceptible to the noncapsulated strain. This was confirmed in several additional tests, some of which are summarized in TABLE 4. Increased susceptibility was indicated by increased mortality and shorter death times. Additional study is needed to establish the specificity of this effect.

TABLE 3

IMMUNIZING VALUE OF FRACTION I IN GUINEA PIGS CHALLENGED WITH A CAPSULATED AND A NONCAPSULATED STRAIN

Challenging strain*	Deaths/total	
	Unvaccinated	Vaccinated with Fraction I
Alexander (Fraction I+)	7/7*	0/5
M23 (Fraction I−)	3/7	6/6

* Organisms (10^5) injected intradermally.

TABLE 4

INCREASED SUSCEPTIBILITY OF GUINEA PIGS VACCINATED WITH FRACTION I TO INFECTION WITH STRAIN M23

Organisms per guinea pig	Route of challenge	Day of death postchallenge	
		Unvaccinated	Vaccinated with FI
10^6	Intraperitoneal	15th 19th	7th 12th
10^5	Intraperitoneal	12th	7th
10^4	Intraperitoneal	18th survived survived	7th 6th 9th 11th
10^5	Intracutaneous	survived	17th
10^4	Intracutaneous	survived	13th
10^3	Intracutaneous	survived survived	14th 25th survived

Several properties, which have been associated with virulence by various workers, are not listed in TABLE 1. Colonial morphology has been correlated with virulence by Soviet workers,[44,3] who state that virulent cultures give rough colonial types and that dissociation into the smooth form is accompanied by a loss of virulence. Eisler[45,46] has described smooth and rough forms of both virulent and avirulent cultures and has observed antigenic differences between smooth and rough forms. Crumpton and Davies[47] have described an antigen associated with the smooth colonial form and possibly contributing to the survival of smooth organisms *in vivo*. Additional studies on colonial variation have been reported recently by Wake.[48,49] The impression was gained several years ago in our laboratory that naturally occurring mutations to streptomycin resistance resulted in loss of virulence if smooth morphology (of the smooth virulent parent) was not retained (A. W. Andrews and M. J. Surgalla, unpublished data). Subsequent observations indicated that rough avirulent streptomycin resistant mutants have lost the calcium requirement, while a group of smooth mutants showing reduced virulence, obtained from the Yokohama strain, were nonpigmented and could be made fully virulent for mice by injection of iron (R. R. Brubaker and M. J. Surgalla, unpublished data). The voluminous literature on this subject is confusing, and any clear-cut relation of colonial morphology to virulence will depend on future work. The finding that virulent strains are more capable than avirulent of proliferating in the chick embryo[1] deserves further investigation.

One of the most interesting mutations commonly observed in our laboratory apparently involves the simultaneous loss of virulence, the calcium requirement, the ability to produce V and W antigens, and sensitivity to glucose. Virulent strains appear to be unstable, "mutating" at a high rate (approximately 10^{-3} to 10^{-4}) to avirulence. Such high "mutation rates" have usually been found to involve various genetic transfer mechanisms rather than classical mutations. Segregating diploids and heterogenotes are examples of situations that give the appearance of having extremely high mutation rates. An example (not involving virulence) may be cited in an unstable strain of *E. coli* that lost, at the rate of about 1 per 1000 cell divisions, 3 cistrons implicated in the metabolism of lactose (governing respectively the synthesis of β-galactosidase, β-galactoside permease, and the repressor assuring regulation of the system).[50] This strain of *E. coli* was a heterogenote, a recombinant possessing two parental alleles of the Lac region, one allele having been carried in by an infectious particle, the F factor. This example of incorporation of a segment of bacterial chromosome in the sexual episome is reminiscent of transduction by phage and has led Jacob and Adelberg[50] to believe that all elements of the bacterial chromosome can become part of an episome and share in its properties. Thus, of the three examples cited in the definition of episomes (by Jacob and Wollman[51]) two (the prophages and F factor) have been demonstrated to incorporate and transfer segments of bacterial chromosome, while the third example (the colicinogenic determinants) may also act as infective particles[52] but have not yet been reported to transfer bacterial chromosomal segments. Since neither transduction nor sexual conjugation has been demonstrated in *P. pestis*, the pesticin described by Ben-Gurion and Hertman[53,54] was the only known property of *P. pestis* whose genetic determinant might be expected to be capable of acting as an infective particle and to function as an episome. A study of plague bacteriocins was undertaken recently to determine whether any bacterial properties could be associated with bacteriocin determinants.

It was assumed that other plague bacteriocins in addition to the original pesticin described by Ben-Gurion and Hertman would be found, and preliminary observations have established their existence (FIGURE 1). Production of pesticin I (assumed to be identical with the originally described pesticin) is demonstrated on the left, and a second pesticin designated pesticin II is shown on the right. Of 3 colonies (virulent *P. pestis* strain Alexander, avirulent *P. pestis* strain A-12, and virulent *P. pseudotuberculosis* Thal I [2]) spotted on Difco blood agar base (BAB), only the first produced pesticin I, effective against the indicator strain Thal I (2). All three colonies produce a second bacteriocin designated pesticin II, effective against the A-12 strain as indicator.

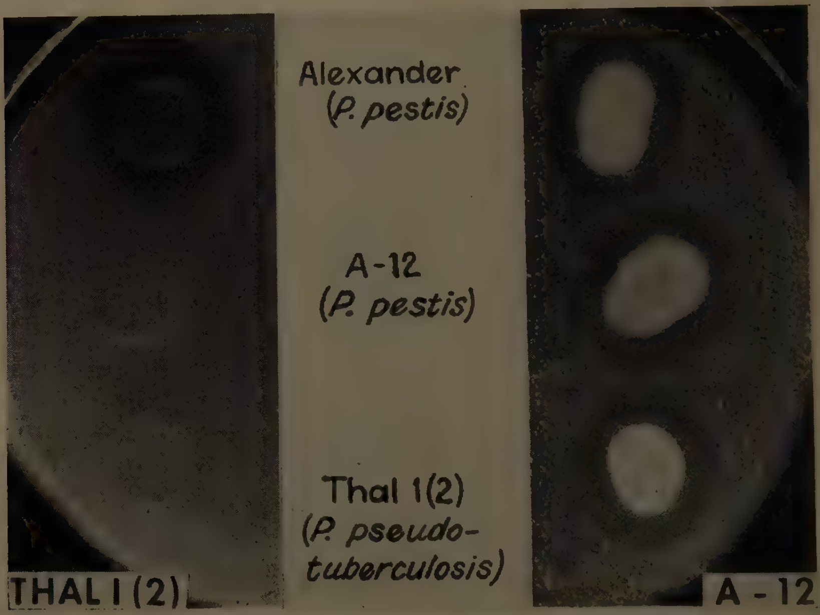

FIGURE 1. Demonstration of bacteriocins produced by *P. pestis* and *P. pseudotuberculosis* Colonies were spotted on BAB (Difco) containing additional 0.1 per cent glucose, 0.04 per cent Na$_2$SO$_3$, and 0.1 *M* CaCl$_2$. After 48 hours incubation at 26° C., the plates were treated with chloroform vapor and overlayed with *P. pseudotuberculosis* Thal I (2) or *P. pestis* A-12 cells contained in 5 ml. BAB. The above inhibition is observed after subsequent incubation at 37° C. for 30 hours. Only the Alexander strain produces a bacteriocin active against *P. pseudotuberculosis*; this has been designated pesticin I and is presumed to be the original pesticin described by Ben-Gurion and Hertman. All three colonies produce a second bacteriocin designated pesticin II active against the A-12 strain.

The activity of both pesticins is enhanced by Ca^{++}; Mg^{++} antagonizes the Ca^{++} effect. Calcium enhances production of pesticin II and depresses pesticin I; Mg^{++} has the opposite effect. All *P. pestis* and *P. pseudotuberculosis* strains tested appear to produce a substance inhibitory to pesticin I. Smooth colonies produce larger pesticin II zones than do rough colonies. The possibility is under study that pesticins may be involved in certain growth-suppression effects, selective for either virulent or avirulent cells, that have been observed.

Concluding Remarks

Some of the bacterial properties considered in the foregoing discussion are certainly involved in pathogenesis; others may prove to be associated with virulence by mere coincidence. A number of questions can be formulated for consideration in future work.

What are the metabolic peculiarities of virulent cells reflected by the calcium requirement and by production (and possible function) of the VW antigens? These two bacterial properties appear to be as closely associated with each other as they are with virulence. Is there a common metabolic basis for these two properties and virulence (assuming the other required factors, such as toxin, are present)? The lethal glucose effect and the ability to grow in macrophages may also be reflections of the same common metabolic peculiarity.

What are the genetic mechanisms controlling the "mutation" resulting in simultaneous loss of virulence (VW) antigens, the calcium requirement, virulence, and possibly also glucose sensitivity and ability to grow in macrophages?

What is the biochemical basis for Ca^{++}-Mg^{++} interactions affecting growth, production of virulence antigens, and production and activity of bacteriocins? If the effects on pesticins I and II have the same metabolic basis as the effects on growth and production of virulence antigens, then these pesticins are related to virulence.

What are the bacterial properties associated with production of or sensitivity to bacteriocins?

What is the nature and mode of action of the toxin (or toxins) as it occurs in the infection and causes death of the mouse and guinea pig?

What is the basis of the immunoparalysis production by Fraction I, and does it exert a general suppressing effect on antibody formation?

What are the salient interactions at the cellular level between the bacterium and the host and where do they take place? For example, are the major determinants of virulence concerned more with resistance to destruction by host defenses or with ability to utilize the host environment for growth? The validity of assay methods for determining the role of any biochemical property in pathogenesis is dependent on these studies.

In conclusion, the contributions to plague research during the past six years have provided an opportunity for comparative study of several different bacterial properties that are related in various ways to each other as well as to virulence. A number of specific and practical experimental approaches are now available that promise to bring our understanding of the pathogenesis of this disease much closer to a biochemical level.

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THE EFFECT OF TEMPERATURE ON THE SYNTHESIS OF VIRULENCE FACTORS BY *PASTEURELLA PESTIS*

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Sokhey and Habbu reported in 1943[1] that the limiting temperatures for the growth of *Pasteurella pestis* were −2° C. and +45° C. with an optimum growth temperature of 27° to 28° C. Since that time only a few publications have appeared correlating the growth temperature to the synthesis of factors that may be implicated in conferring maximal virulence. Meyer and his associates have indicated that the envelope or capsular substance designated as Fraction 1 (F1), produced at 37° C., rendered *P. pestis* resistant to phagocytosis.[2] Burrows and Bacon have reported that incubation at 37° C. was necessary to render virulent *P. pestis* cultures previously grown at 28° C. resistant to phagocytosis by mouse neutrophiles in the absence of demonstrable encapsulation and for the synthesis of V and W antigens that they associated with virulence.[3,4]

Fukui *et al.* have demonstrated that the virulence of cultures grown on agar slants at 37° C. was usually greater than similar cultures grown at 26° C.[5] and was comparable in virulence to cultures grown *in vivo* (unpublished data). The results of subsequent studies comparing the virulence of cultures grown at 5° C., 26° C., and 37° C. revealed a direct correlation between the growth temperature and virulence. The virulence of cultures grown at 5° C. was consistently depressed, but it could be fully restored during a short period of incubation at 37° C. in the absence of apparent cellular multiplication.

Materials and Methods

Two strains of *P. pestis* were employed: Alexander, isolated from a fatal human case in New Mexico in 1949, and M23, a Fraction 1-deficient strain isolated by Burrows in 1957.[6]

The inocula for 5° C. cultures were initiated by inoculating 50 ml. of heart infusion broth (Difco) supplemented with 0.25 per cent xylose and 0.06 per cent $MgSO_4 \cdot 7H_2O$ in a 500 ml. Erlenmeyer flask with the growth from a blood agar base slant (Difco), which had been incubated at 26° C. for 24 hours. The cultures were grown with aeration on a reciprocating shaker placed in a 5° C. cold room. After 4 days of incubation, 10 ml. of this culture was used to inoculate 50 ml. of heart infusion broth (HI) that was also permitted to incubate at 5° C. for 4 days. The viable number of cells was determined by the pour-plate method using blood agar base supplemented with 0.1 per cent glucose and 0.04 per cent sodium sulfite. The plates were incubated for 48 hours at 26° C. before counting. The total number of cells was determined with the use of a Petroff-Hausser and Helber counting chamber.

In order to study the restoration of virulence by incubation at 37° C., a 2-ml. aliquot of the 5° C. culture was removed and the remaining culture was placed on a reciprocating shaker in a 37° C. incubator. The mouse intraperitoneal virulence titrations were determined by challenging Swiss-Webster mice

(18 to 22 gm.) with 0.2 ml. of appropriate serial dilutions of the culture in HI broth. A minimum of 6 doses with 10 mice per dose was used for each titration. The mouse LD_{50} based on the numbers of mice dying during a 10-day period was calculated by the method of Litchfield and Wilcoxon.[7]

The guinea pig intratracheal, intracutaneous, and intraperitoneal virulence titrations were performed by challenging the Hartley strain of guinea pigs (350 to 500 gm.) with 0.1 ml. of appropriate serial dilutions. A minimum of 6 doses with 10 guinea pigs per dose was used for each virulence titration. Intratracheal inoculations were made after exposing the trachea of guinea pigs anesthetized with nembutal by a standard surgical technique. The guinea pig intraperitoneal and intratracheal LD_{50} values were calculated from the numbers of animals dying within 21 days postchallenge, and the intracutaneous LD_{50} value was calculated from the numbers of animals dying within 30 days postchallenge.

Assays for toxin, Fraction 1, V, and W antigens were determined by the gel-diffusion precipitin technique. The development of resistance to phagocytosis was determined by *in vitro* techniques previously described.[8]

Results

Upon preliminary investigation of the rate of growth of *P. pestis* at 5° C., it was learned that 3 to 4 days of incubation were required to obtain a log increase in the number of viable cells (FIGURE 1). The results of a representative experiment performed to demonstrate the effect of growing cultures at 5°, 26°, and 37° C. on the syntheses of antigens associated with virulence and its correlation to the expression of virulence in terms of mouse LD_{50} values are presented in TABLE 1. Of the 4 antigens assayed by the gel-diffusion technique, only toxin was detected in the cultures grown at 5° or 26° C. In addition to toxin, F1, V, and W antigens were detected in the cultures grown at 37° C. The virulence of the culture grown at 37° C. was 100 times greater than the culture grown at 5° C. The virulence of the culture grown at 26° C. was approximately 8 times greater than that of cultures grown at 5° C., even though the antigenic structures of these cultures (for the 4 antigens tested) were similar.

Quantitative analysis of the cultures grown at 5°, 26°, and 37° C. for numbers of virulent and avirulent cells in the population, by single-colony isolation and testing the virulence of 20 colonies picked at random, indicated that 20 colonies picked from each culture were virulent. Therefore the results of the foregoing experiments indicated that the lowered virulence of the cultures grown at 5° C. was not due to the selective growth of avirulent cells but probably due to the inactivity of enzymes at 5° C. required for the synthesis of factors necessary for the expression of maximal virulence.

In order to substantiate the latter hypothesis, experiments were performed to demonstrate the restoration of maximal virulence to cultures grown at 5° C. by incubation at 37° C. A culture previously grown at 5° C. was incubated at 37° C. with aeration, and samples were withdrawn at 2-hour intervals and assayed for "virulence antigens," resistance to phagocytosis, viable and total number of cells, and mouse intraperitoneal virulence titrations (FIGURE 2). The results indicated that, within 2 hours, a slight increase in virulence could be detected and, within 8 hours, about a 100-fold increase in virulence could be demonstrated. Also the synthesis of F1, V, and W antigens, as well as the development of resistance of phagocytosis, could be correlated directly to the increase in virulence. During the 8 hours of incubation, no apparent increase in numbers of viable or total cells could be detected. These results would also strongly imply a phenotypic shift in virulence and not a selection of genotypically virulent cells as might be encountered in a growing culture, especially in the case of *in vivo* growth where the host defense mechanism might eliminate avirulent cells.

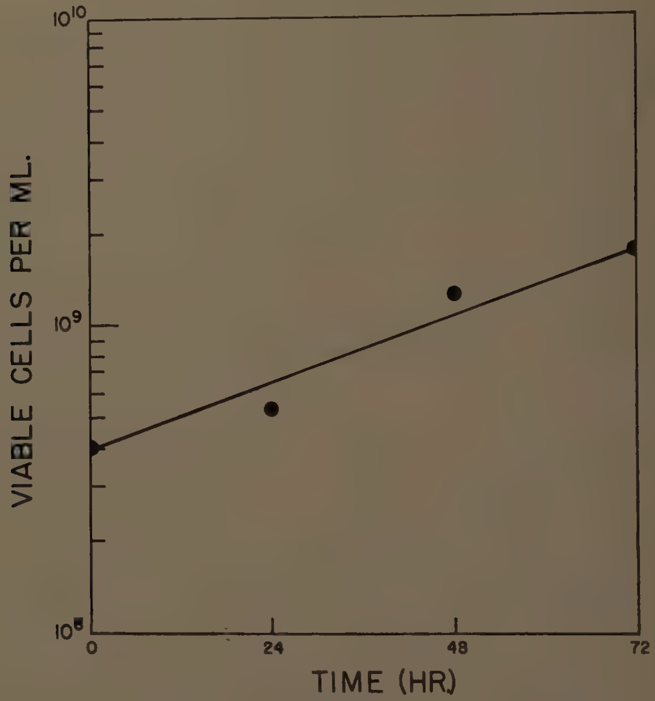

FIGURE 1. Growth of *P. pestis* at 5° C.

TABLE 1

THE CORRELATION OF GROWTH TEMPERATURE WITH VIRULENCE AND PRODUCTION OF DETECTABLE ANTIGENS BY *PASTEURELLA PESTIS*, ALEXANDER

Temperature (°C.)	Antigens				Mouse IP LD_{50} (95% C.L.)
	Toxin	F	V	W	
5	+	0	0	0	500 (210–1200)
26	+	0	0	0	38 (16–88)
37	+	+	+	+	5 (3–11)

The foregoing virulence restoration, demonstrated with the classically virulent Alexander strain, was also observed with the M23 strain. These observations confirm the reports of Burrows[6] that the V and W antigens coupled with the toxin confer maximum virulence, at least for the mouse, and that F1 may not be necessary for the expression of virulence for the mouse.

In an attempt to elucidate further the temperature dependency for the synthesis of virulence factors and possibly the biological function of these factors, virulence titrations were performed with a more resistant test animal, guinea pigs, challenged via the intratracheal, intraperitoneal, and intracutaneous routes. The culture was prepared in a manner similar to those described previously, and virulence titrations were performed with the culture grown at 5° C. and after it had been incubated at 37° C. for 3 and 6 hours. The results of these titrations indicated approximately a "148-fold increase" in virulence within 6 hours if the guinea pigs were challenged via the intraperitoneal route, a 256-fold increase in virulence via the intracheal route, and a 7-fold increase in virulence when challenged via the intracutaneous route (FIGURE 3). Although an inverse correlation of LD_{50} values to the synthesis of virulence antigens was

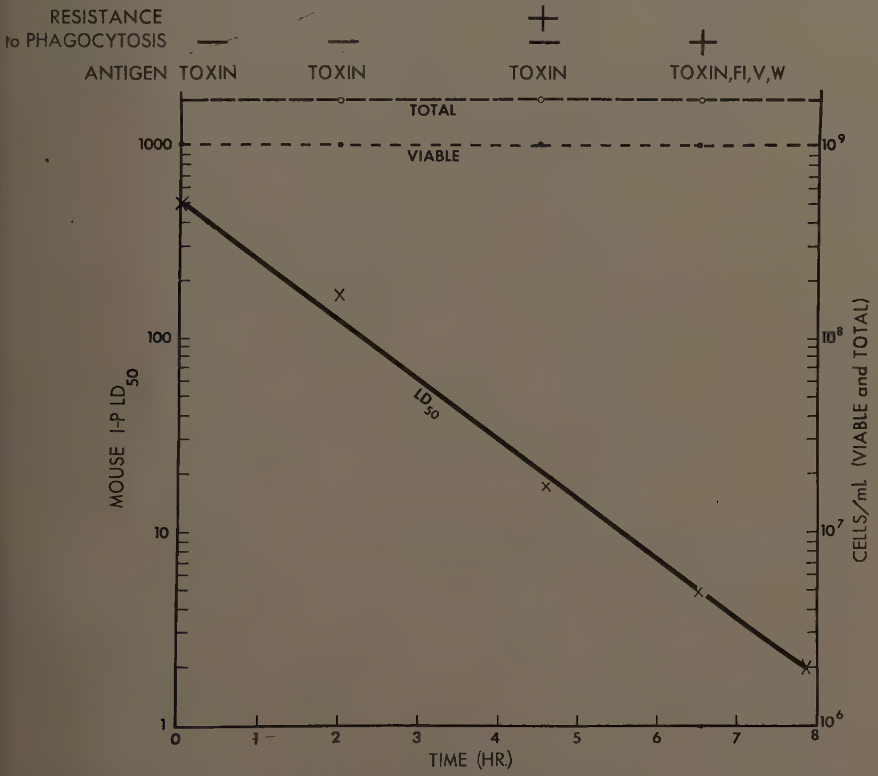

FIGURE 2. Phenotypic changes in virulence of *P. pestis* related to temperature (5° → 37° C.).

noted, no apparent resistance to phagocytosis by guinea pig neutrophiles could be detected by the *in vitro* phagocytosis tests. However, this strain of *P. pestis* Alexander had been demonstrated to become resistant to phagocytosis when grown under certain conditions at 37° C. or when harvested from the peritoneal exudate of guinea pigs (W. A. Janssen, unpublished data). Similar virulence titrations performed with the M23 strain that synthesized V and W antigens during the 6 hours of incubation at 37° C. generally manifested a trend toward an increase in virulence for the guinea pig, but complete restoration was not attained within 6 hours of incubation.

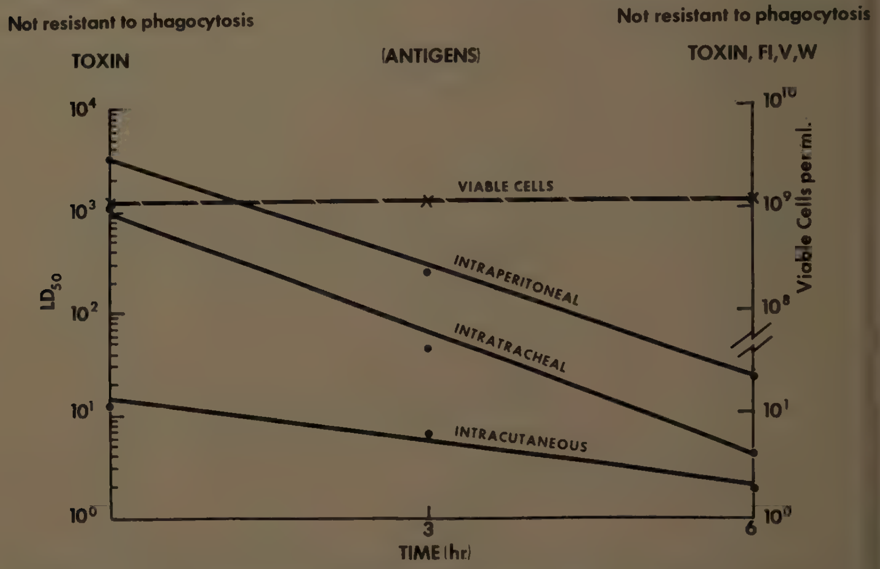

FIGURE 3. The enhancement of virulence for guinea pig in nonmultiplying cells of *P. pestis* at 37° C.

TABLE 2
THE EFFECT OF CHLORAMPHENICOL ON THE RESTORATION OF VIRULENCE OF *PASTEURELLA PESTIS*

Chloramphenicol* (µg./ml.)	Viable cells per ml.	Antigens†			LD_{50} (95% confidence limits)‡
		F1	V	W	
Culture grown at 5° C.:	6.0×10^8	—	—	—	2400 (1600–4000)
5° C. culture incubated 6 hours at 37° C.:					
None	8.6×10^8	Tr	+	+	9.0 (5.7–14.0)
10.0	4.4×10^8	—	—	—	5000 (3000–8800)
2.0	5.3×10^8	—	—	—	340 (220–550)
1.0	4.4×10^8	—	Tr	Tr	24 (17–38)
0.4	6.4×10^8	—	+	+	20 (12–33)
0.1	6.6×10^8	Tr	+	+	9.0 (5.7–14.0)

* Chloramphenicol from Parke Davis, Detroit, Mich.
† Assayed by gel-diffusion; —, not detected; Tr, trace.
‡ Mouse intraperitoneal virulence titrations.

These results might permit us to speculate that the host defense mechanisms were more effective in the peritoneal cavity and the respiratory tract than in the dermis. The plague microorganisms were apparently not readily accessible to the host defense mechanisms in the dermis, and the cells from cultures grown at 5° C. were able to synthesize the necessary virulence factors *in situ* at the body temperature of the guinea pig (38.8° C.), thus rendering them resistant to the host defense mechanisms. Since the virulence for the guinea pig was not fully restored in the M23 strain by incubation at 37° C., it must be concluded, as was done by Burrows,[6] that F1 plays a very significant role in the expression of virulence by *P. pestis* for the guinea pig.

Since the requirement for protein synthesis was suggested by the synthesis of temperature-dependent virulence antigens, experiments were performed to determine the effect of chloramphenicol on the restoration of virulence. The results of several experiments indicated that 10 μg. per ml. of chloramphenicol inhibited completely the synthesis of F1, V, W antigens, and the restoration of virulence without affecting the viability of the cells (TABLE 2).

The results presented in this paper point out several interesting factors relating the physiology of *P. pestis* to the synthesis of virulence factors, variability in susceptibility of different species of test animals, and the marked differences in the host response depending upon the anatomical site of inoculation. The temperature-dependent phenotypic shift in virulence offers a useful assay system for studying the physicochemical requirements for the synthesis of virulence factors in contrast to those required for growth.

Summary

The results of these studies demonstrated the restoration of maximum virulence to cultures that had undergone a phenotypic loss of virulence during growth at 5° C. This restoration of virulence was accomplished by incubation at 37° C. for a short period of incubation (6 to 8 hours) in the absence of significant cellular multiplication. The increase in virulence was correlated with the ability to detect known virulence antigens and, to a lesser extent, resistance to phagocytosis.

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THE BIOCHEMISTRY AND PHYSIOLOGY OF THE PLAGUE MURINE TOXIN

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Our work on microbial toxins started in 1952 when the United States Army was interested in plague because of the possibility, during the Korean conflict, that United States troops might be exposed to it in Manchuria, which has been the historical breeding place of pandemics of plague.

Investigations on plague toxin have been prompted by the observations of McCrumb *et al.*,[1] in Madagascar, that antibiotics administered 36 to 48 hours after onset of the disease failed to save patients in spite of the fact that on autopsy the organs and blood were sterile. Furthermore, such plague victims at autopsy showed typical symptoms of so-called toxic deaths.

Our primary objective from the start of this work has been to isolate and purify this toxin and to study the mechanism of its action at an enzymatic level. At the time this work was started, the plague bacillus was known to contain several distinct and readily separable antigenic components.[2] Two of these, the envelope antigen and the toxin, have been studied in some detail, particularly in regard to their role in immunity. The recent work of Burrows and his co-workers[3] and Lawton and his co-workers[4] revealed a number of additional significant antigenic components, but our interests have remained in the toxin that *Pasteurella pestis* produces: the toxin responsible for the death of mice or rats and consequently referred to as the "murine" toxin.

Our initial studies dealt with the purification of the toxin by chemical means.[5] These involved extraction of the toxin from acetone-dried cells with 2.5 per cent sodium chloride, followed by ammonium sulfate and isoelectric precipitations, treatment with magnesium chloride and alcohol precipitation, calcium phosphate gel absorption and elution, and chloroform extraction. The product thus obtained behaved as a homogenous protein in the ultracentrifuge and electrophoresis cells and was found to be free of nucleic acids, carbohydrates, and capsular material. However, the purity of this material, when ascertained by the very sensitive immunological gel diffusion precipitation reactions of Oudin and Ouchterlony, showed at least two and, often, three or more individual zones of precipitation.

Since our major objective had been to determine the mechanism of action of the toxin at an enzymatic level, it became imperative to obtain preparations that were "pure." The high resolving power for fractionating protein mixtures afforded by continuous paper electrophoresis led to the employment of this technique for the final purification of the toxin.[6] The continuous-flow, hanging-curtain electrophoresis apparatus developed by Karler[7] has been utilized in the isolation from crude lysed cell cultures of small quantities of toxin that exhibited one band in the very sensitive gel diffusion precipitation reactions.

Starting with a material with an intraperitoneal LD_{50} of 18 μg. for 16- to 18-gm. mice, after several passes through the Karler electrophoresis apparatus a material was obtained with an LD_{50} of less than 0.1 of 1 μg. On the basis of sedimentation and diffusion data, the molecular weight of this toxin was calculated to be approximately 74,000. The toxin thus obtained contained 15 per cent nitrogen, no carbohydrate or phosphorus, but significant amounts of sulfur. The carbon and hydrogen content was 46.47 per cent and 6.62 per cent respectively.

In an effort to determine whether there is an unusual component in the toxin that is responsible for its high toxicity, a detailed elemental and amino acid analysis was performed on highly purified samples of the toxin.[8] Eighteen amino acids and a number of elements were identified and on a dry-weight basis over 98 per cent of the toxin molecule was accounted for by the organic analysis, including ammonia and ash content. Aside from the high proportions of acidic amino acids, no unusual organic component that might be responsible for its toxicity was detected.

A series of immunological experiments with the pure material yielded the following information: antitoxin can be readily prepared in rabbits with adjuvant; the antiserum thus obtained flocculates, hemagglutinates, and fixes complement with toxin and toxoid; immunization with formalin-treated toxin protects mice against 60 to 80 LD_{50} of the toxin when the animals are challenged intraperitoneally; only a few LD_{50} of toxin are neutralized when toxin-antitoxin mixtures are administered intravenously to mice; reactions of pure *P. pestis* toxin with various plague antisera against a number of strains and the reaction of various antigenic components against specific antiserum reveal that this bacterium contains only one murine toxin.

In an effort to determine the mechanism of action of plague toxin *in vitro*, small amounts of the toxin were added to microbial extracts, crude mouse and rat liver, and heart homogenates oxidizing a variety of substrates.[9] Initial experiments revealed that plague toxin specifically inhibited the oxidation of α-keto acids. For example, whereas plague toxin inhibited the oxidation of α-ketoglutaric and pyruvic acids, it showed a significantly lesser effect on such acids as succinate and fumarate. It has been possible to reverse this inhibition on the addition of DPN and not triphosphopyridine nucleotide (TPN).

To test the action of toxin on a higher level of organization, from the standpoint of enzymatic structure and closer to what may be expected to occur in the animal and at the same time maintaining an *in vitro* system, a study was undertaken to test the effect of pure toxin on mitochondria that are considered to be the active sites of respiration in animal tissues. Here, a very interesting observation was made,[10] an observation that may provide a clue to the understanding of the susceptibility and resistance of animal species to biological poisons. An association was found to exist between the ability of plague toxin to inhibit the respiration of mitochondria from certain species and susceptibility of these animal species to the action of the toxin. It has already been pointed out that the murine toxin kills mice and rats. It has no effect, even in enormous concentrations, on the toxin-resistant rabbit, dog, monkey, or guinea pig. It was, therefore, of great interest to learn that, whereas toxin inhibited the respiration of heart mitochondria obtained from the toxin-sensitive rat and mouse, it had no effect on the respiration of heart mitochondria from the toxin-resistant rabbit, dog, monkey, or chimpanzee. Further, only exogenous respiration was inhibited; endogenous respiration and oxidative phosphorylation were unaffected. This inhibition is specific for plague toxin in so far as bovine serum albumin, representing another protein, and the Vi and O lipopolysaccharide antigens, for example, do not interfere with mitochondrial respiration. Unlike the results obtained with microbial extracts,[6] toxin treated in any manner that makes it atoxic for the animal also results in a loss of the capacity to inhibit mitochondrial respiration. Minute physiological concentrations of toxin are required to demonstrate the respiratory inhibitions. A correlation therefore exists between the toxicity of the toxin molecule *in vivo* and its ability to inhibit mitochondrial respiration.

Our present hypothesis is therefore that inhibition of mitochondrial respiration may be an explanation for the action of plague murine toxin *in vivo.*

To test this hypothesis, further attempts were made to correlate the *in vitro* changes in the intact animal. It was assumed that, since rat and mouse heart mitochondria were found to be susceptible to the action of plague murine toxin, certain alterations in their myocardial physiology might be expected that could possibly be detected by electrocardiographic measurements. It was found that alterations in the S-T segment of the cardiogram occurred within 60 min. after the injection of lethal or sublethal doses of toxin and prior to any changes in hematocrits or blood pressures. It was also interesting to learn that in surviving animals receiving sublethal doses of toxin, electrocardiographic changes that are observed initially are no longer evident after 24 to 48 hours or after the animal has completely recovered. These changes may be specific for plague toxin as evidenced by the finding that similar changes do not occur in rats dying from hemorrhagic shock, hypoxia, or in toxic deaths from *Escherichia coli* endotoxin. In addition the rabbit, which is not susceptible to the murine toxin, did not show any abnormality of the electrocardiographic tracing when given this toxin in doses of up to 50 μg.

The basic observation in this work, therefore, is that here is a situation where we can correlate the susceptibility and resistance of an animal to a biologic poison with the inhibition of a specific enzymatic reaction.

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BIOCHEMICAL PROPERTIES OF VIRULENT AND AVIRULENT STRAINS OF BRUCELLAE*

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To place the brucellae within the framework of this monograph and to relate any observed *in vitro* biochemical differences to virulence, we shall consider several important points: (1) smooth brucellae multiply intracellularly, and (2) smooth strains of low virulence and of high virulence have equal toxicity. This leads to a third point: Is the primary attribute of virulence in the brucellae the ability to grow within certain cells of the host?

The reports leading to the conclusion that the brucellae grow intracellularly have been reviewed by Spink (1956). Within the past 10 years several groups (Schaffer *et al.*, 1953; Holland and Pickett, 1956; Pomales-Lebron and Stinebring, 1957; Braun *et al.*, 1958; Freeman and Vana, 1958) have studied the interaction of brucellae with monocytes and other tissue cells maintained *in vitro*. For example, Spink and his associates (Schaffer *et al.*, 1953) have shown that it is the intracellular localization of brucellae that protects these organisms from certain antibiotics as well as antiserum.

Experiments on toxicity have been conducted by injecting into mice either large numbers of killed whole cells of brucellae or extracted endotoxin. Spink and Anderson (1954) prepared endotoxin from the three common brucellae species by the Boivin technique, and these preparations showed equal toxicity as measured by death when injected intravenously into mice. Toxin extracted from a strain of *B. abortus* of low virulence was just as lethal as that from a highly invasive strain of *B. melitensis*. No lethal toxin could be prepared from a mucoid, nonsmooth strain of *B. abortus*, indicating that endotoxin is associated only with the smooth type. Gilardi (1956), working with D. T. Berman at the University of Wisconsin, observed nearly equal toxicity as measured by death of acetone-killed *B. suis* strains differing in virulence when injected intraperitoneally into mice (TABLE 1). These cultures were all smooth or smooth-intermediate strains derived from the highly virulent strain SS-2a. The mutants were selected *in vitro*, tested for virulence (Simon, 1955), and then tested for toxicity in mice (Gilardi, 1956). Thus these closely related cultures had nearly equal toxicity regardless of virulence.

Since smooth strains have about the same toxicity, the strains of low virulence must be limited in their ability to grow in the host. The ability to multiply intracellularly then appears to be a crucial point in determining the virulence of a particular strain. That this is true is indicated by results from several laboratories. Mice injected intravenously with living cells of virulent strains of *B. abortus* died in 1 to 2 days, whereas mice injected with strains of low virulence died in 6 to 7 days (Spink and Anderson, 1954). In light of their results with extracted endotoxin, these investigators explained their data

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by postulating slower growth rate of the strains of low virulence in the host tissues. The "whole mouse" culture technique that Berry (1956) has used in studying *Salmonella typhimurium* infections could be used in this type of experiment with brucellae.

The tissue culture work done with brucellae of different virulence shows differences in intracellular growth. Whether tested with chick embryo fibroblasts (Holland and Pickett, 1956), guinea pig or mouse monocytes (Braun *et al.*, 1958; Holland and Pickett, 1958), or HeLa cells (Shepard, 1959), the strains of low virulence are limited in their ability to multiply intracellularly, whereas the virulent strains can multiply extensively in these systems. It is not known what the basis of the selective effect of the intracellular environment is, nor is it known if the same effect is involved in the various tissue systems mentioned. Freeman and Vana (1958) present data that are somewhat contradictory, but their experiments were done under conditions different from that of the authors cited above. Clearly more knowledge is required about the physiology of the host-parasite interaction. However, *in vitro* investigations on the metabolism of strains of low and high virulence should yield information that can give directions to studies on intracellular growth.

TABLE 1

COMPARISON OF GUINEA PIG VIRULENCE AND MOUSE TOXICITY OF STRAINS OF *BRUCELLA SUIS**

	Strain	Pathogenicity for guinea pig (progressive infection)	Mouse LD_{50} of acetone-killed cells (mg.)
Experiment No. 1	SS-2a	High	7.4
	SS-18b	Low	6.5
	SS-11	Intermediate	5.3
Experiment No. 2	SS-2a	High	9.7
	SS-11	Intermediate	7.7
	SS-18a	Low	7.5
	SS-3	Low	9.0

* Gilardi (1956).

The smooth-rough variation of brucellae has been thoroughly described (Huddleson, 1952; Braun, 1950). The biochemical basis for this variation is still uncertain (Gerhardt, 1958); however, the suppression of smooth cells by accumulation of D-alanine in the culture medium (Goodlow *et al.*, 1950) and the effects of a low oxygen tension (Sanders and Huddleson, 1956; Altenbern *et al.*, 1957*b*) figure prominently in these changes. The appearance of the colonies themselves (Huddleson, 1952) suggests differences in the outer cell constituents. Staining the colonies with dilute crystal violet makes this visual difference more apparent (White and Wilson, 1951). Stinebring and Braun (1959) also have shown that rough and mucoid *B. abortus* strains are not susceptible to lysis by brucellaphage, whereas smooth and smooth intermediate strains are lysed. Some pertinent observations on biochemical differences between smooth and nonsmooth strains (and by implication of differing virulence) are:

(1) Rough type cells exhibit a greater rate of pantothenate uptake than do smooth type cells (Altenbern *et al.*, 1957*a*).

(2) At reduced oxygen levels rough types grow more rapidly than smooth types (Sanders and Huddleson, 1956; Altenbern *et al.*, 1957*b*). The latter authors consider that there may be a difference in terminal hydrogen transport systems between smooth types and rough types.

(3) Rough type *B. melitensis* are more susceptible to a lysozymelike enzyme from monocytes in the presence of glycine than are smooth type cells (Ralston *et al.*, 1960).

Other differences between rough and smooth cells are: nonsmooth cells are agglutinated by normal rabbit serum and by dilute acriflavine, whereas smooth cells are not agglutinated; smooth cells are agglutinated only by antibrucella serum, but nonsmooth cells have varying responses depending on cell type; mucoid and rough cells do not contain endotoxin.

Now turning to the physiology of smooth type cells where gross surface differences may not be a problem, the nutrition and metabolism of smooth brucellae have been studied extensively (Gerhardt, 1958; Spink, 1956; Kuwahara *et al.*, 1957), but little work has been directed toward the relationship of physiology to virulence. The amino acid and nucleic acid composition of *in vitro* grown *B. abortus* has been studied by Tepper and Wilson (1958*a*) to determine if differences in composition could be correlated with the virulence of the organism. No difference was found either quantitatively or qualitatively in amino acid composition in a strain of relatively high virulence and one of low virulence. These same strains also contained essentially the same quantity of total DNA and RNA. Tepper and Wilson (1958*b*) also studied the fixation of CO_2 by strains of low and high virulence and found that there was no difference in the total incorporation of $C^{14}O_2$ by these strains; no difference was found in the distribution of C^{14} fixed into the amino acids; nor was there any difference in the percentage composition of the C^{14} in chemical fractions of the cells after both short and long-term exposures.

Huddleson and Stahl (1943) have reported that the catalase activity of the *Brucella spp.* is directly related to their virulence. This relationship holds true within the species only, because each species has a different range of catalase activity (TABLE 2). The catalase activity within each species decreases with a decrease in virulence. This decrease occurs with a dissociation to mucoid or rough variants or in a smooth strain of less virulence. The authors did not indicate how an increased catalase activity contributes to virulence. These results have been confirmed by Seelemann *et al.* (1951) and Parnas and Melichevskii (1958). Some results that at first appear to be to the contrary have been reported by Waring *et al.* (1953). *B. suis* mutants were derived that did not require iron and were nearly catalase-free when grown in an iron-free medium. These cells were more invasive in mice and guinea pigs than was the parent catalase-producing strain. There are no data presented as to the catalase activity of the strains recovered from the animals. Also these authors report that, when the iron deficient mutants are cultured in an iron-containing medium, the catalase activity becomes that of the parent. We suggest that this same thing happens *in vivo*, although the initial phagocytosis in the host would be of catalase-free cells.

Gerhardt *et al.* (1950), Gerhardt (1949), and Levine (1949) surveyed a number of amino acids and related nitrogen compounds and determined their rates of oxidation by *B. abortus* strain 19, a strain of low virulence. The rate of oxygen uptake with glutamic acid was considerably greater than with any of the other compounds examined. Levine (1949) reported a difference in the effect of capryl alcohol on the oxidative rates of *B. abortus* strains of low and high virulence (TABLE 3). The oxidative rates of strains of high virulence were stimulated over the endogenous, whereas strains of low virulence exhibited an initial stimulation followed by subsequent depression to a level below that of the endogenous. Levine (1949) and Olsen (1951) surveyed a number of other substrates including sugars, alcohols, organic acids, and amino acids with *B. abortus* strains of low and high virulence and found that glutamic acid

TABLE 2

COMPARISON OF THE CATALASE ACTIVITY OF THE SPECIES OF *BRUCELLA**

Species and numbers	Colonial type	Virulence for guinea pig	H_2O_2 decomposed in 30 min., expressed in ml. of 0.1 *N* $KMnO_4$
Abortus 1287E	Smooth	High	7.75+
Abortus 1293	Smooth	High	7.036
Abortus 1296	Smooth	High	8.694
Abortus 1257	Smooth	High	7.919
Abortus 19	Smooth	Low	4.413
Abortus 19	Rough	No	4.453
Abortus 1252	Smooth	No	2.100
Abortus 1252	O-wedge	No	1.320
Abortus 295	Smooth	No	1.470
Abortus 295	Rough	No	2.820
Abortus 45	Intermediate	No	2.563
Abortus 85	Rough	No	3.047
Melitensis 2466	Smooth	High	11.592
Melitensis 2461	Smooth	High	10.498
Melitensis 2459	Smooth	Low	7.231
Melitensis 2458	Smooth	Low	7.237
Melitensis 2469	Smooth	Moderate	9.065
Melitensis 2469	Rough	No	7.980
Melitensis 381	Rough	No	6.822
Suis 1716	Smooth	High	40.785
Suis 1716	Rough	No	28.822
Suis 403	Rough	No	24.342
Suis 1675	Smooth	Moderate	31.350
Suis 1681	Smooth	Moderate	31.577
Suis 1699	Smooth	High	34.269
Suis 1677	Smooth	High	37.528
Suis 1677	Rough	No	25.124
Suis 1707	Smooth	High	38.719
Suis 1710	Smooth	High	38.719
Suis 1616	Smooth	High	40.758
Suis 1716	Rough	No	28.822
Suis 1717	Smooth	No	17.181

* Huddleson and Stahl (1943). Reproduced by permission of *Michigan State College Agricultural Experimental Station Technical Bulletin.*

was the only compound of those tested that showed any appreciable difference in oxidative rate that could be correlated with the virulence of the strain (TABLE 4). Oxidative rates of glutamic acid by *B. abortus* strains of low and high virulence are given in TABLE 5. While as a species, *B. suis* (TABLE 6) has a lower oxidative rate on glutamate than *B. abortus*, the relationship to virulence also holds true in this species (Kamm, 1956; Grogan, 1957). Of the strains listed in TABLE 6, all of the SS21 series were derived from the parent

TABLE 3

THE QUALITATIVE EFFECT OF CAPRYL* ALCOHOL ON THE RESPIRATION OF *BRUCELLA ABORTUS* STRAINS (LEVINE, 1949)

*p*H 6.8; temperature 34° C.

Strain	Guinea pig virulence	Initial stimulation	Subsequent depression
19	Relatively low	+ Slight	+
11	Low	+	+
6980	Relatively low	+	+ Slight
45.20	Relatively low†	+	+ Slight
2308	High	+	−
120	High	+	−
544	High	+	−

* Final concentration M/300.

† Rough variant of a potentially virulent strain.

TABLE 4

$QO_2(N)$ VALUES FOR SEVERAL STRAINS OF *B. ABORTUS**

Substrate	Strain				
	19 (smooth)	19-19-R (rough)	11 (smooth)	6980 (mucoid)	2308 (smooth)
	Virulence				
Virulence	Low	Low	Very low	Low	High
α-Ketoglutarate (M/80)			147		150
α-Ketoglutarate†	85‡		35	24	57
Succinate	191	152	142	164	152
Citrate	0	0	0	0	0
Lactate	177	148	157	331	209
Acetate	208	200	200	175	250
Propionate	0	0	0	0	0
Glucose	313	245	251	504	209
Fructose	212	25	23	90	77
Maltose	48	25	0	19	49
L-Alanine	17	72	66		30
L-Aspartate	18	27	11		21
L-Glutamate	444	427	509	464	128

* Olsen (1951).

† Substrate concentration M/300 unless stated otherwise.

‡ Endogenous rates subtracted.

SS21s-2a that is of high virulence. These mutants were tested for virulence (Simon, 1955) and subsequently tested for rate of glutamate oxidation. The work with these mutants more closely relates glutamate oxidation to virulence than if unrelated strains were used. We have no results with *B. melitensis*. Work reported from another laboratory (Meyer and Cameron, 1959) that was concerned with species differentiation in the brucellae agrees with the results from our laboratory in those cases where virulence of the culture is known.

TABLE 5

RATE OF GLUTAMIC ACID OXIDATION BY *BRUCELLA ABORTUS* IN RELATION TO VIRULENCE*

Strain	Virulence	$QO_2(N)$†
19	Low	444
11	Low	486
6980	Low	441
45.20	Low	312
544	High	111
2308	High	128
Krause-C	High	88
6232-C	High	150

* Olsen (1951).
† Endogenous subtracted.

TABLE 6

RATE OF GLUTAMIC ACID OXIDATION BY *BRUCELLA SUIS* IN RELATION TO VIRULENCE*

Strain	Virulence	$QO_2(N)$†
SS21s-18b	Low	240
SS21s-15	Low	262
3a3d	Low	281
SS21s-2a	High	137
1754	High	91
SS21s-BG	High	136
SR21-GP	High	100

* Grogan (1957).
† Endogenous subtracted.

Another comparison that has been made with strains of low and high virulence is that of the effect of low oxygen tension on the respiration rate. This type of study has been made with avirulent and virulent strains of *Mycobacterium tuberculosis* by Heplar *et al.* (1953) and Guy *et al.* (1953), who found that growth and respiration of avirulent strains were suppressed to a greater extent than were the virulent strains in an atmosphere of 1 per cent oxygen. Our first Warburg experiments at 1 per cent oxygen with brucellae (Kamm and Wilson, 1956) lead us to believe that low virulent strains were depressed in their respiration much more than were high virulent strains. On reducing the quantity of cells in the Warburg vessels (Grogan, 1957) so that oxygen starvation was not a factor, we found no depression of respiration rates of strains of low or high virulence. The effect of low oxygen tension on the respiration of *B. abortus* was further studied with the polarograph using a dropping mercury electrode (Dasinger, 1960). It was shown (FIGURE 1) that the rate of change in oxygen concentration is not affected down to 0.05 ppm of oxygen. This is equivalent to about 0.1 per cent oxygen in the atmosphere above the liquid. It appears that neither the strains of low virulence (11 and 19) nor the strains of high virulence (6232-C and 2308) are affected by the lowest oxygen concentrations measured.

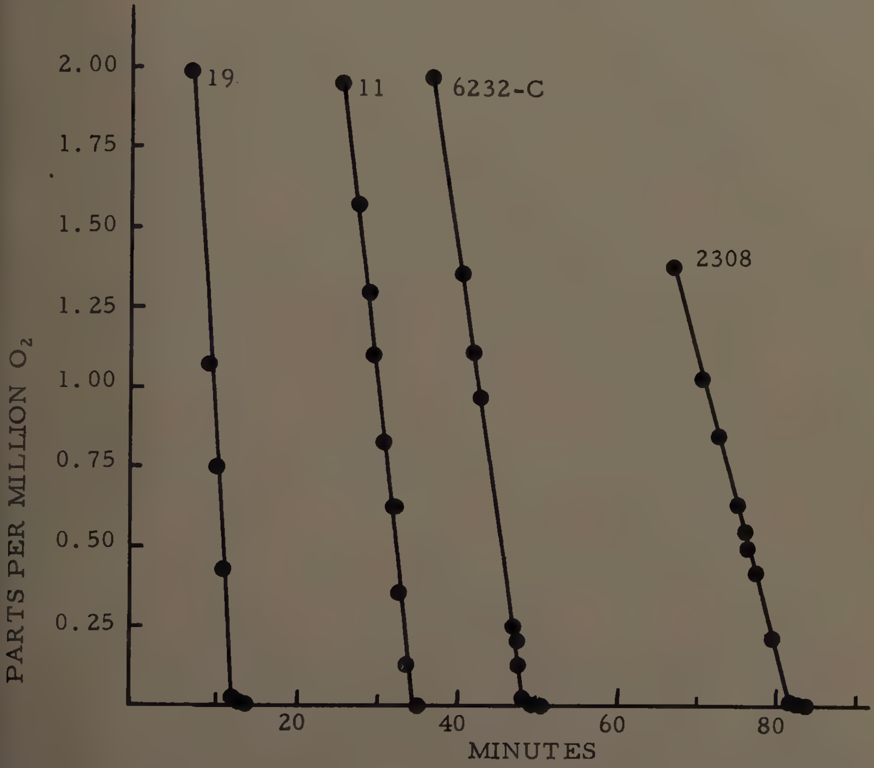

FIGURE 1. Effect of low pO_2 on the rate of glutamate oxidation by strains of *Brucella abortus*

The results presented above show that *B. abortus* and *B. suis* strains of low virulence oxidize glutamate at a high rate, whereas strains of high virulence oxidize glutamate at a much slower rate. Because this relationship has been observed with a large number of strains, it is felt that there may be a connection between the rate of glutamate oxidation and the virulence of the strain. As a first step in understanding this relationship, experiments were conducted to find the basis for the difference. This difference between strains could be explained by several hypotheses:

(1) The rate-limiting factor is the internal metabolism of glutamate. This

in turn leads to two possibilities: different pathways of glutamate oxidation with different activities, and identical pathways but quantitative differences in enzyme concentration.

(2) The limiting factor lies in permeability differences.

(3) A combination of the above.

A study of the pathway of glutamate oxidation in *B. abortus*, strain 11 (low virulence), by Marr *et al.* (1953) indicated that glutamate entered the tricarboxylic acid cycle via α-ketoglutarate that was oxidized to oxaloacetate. The latter compound was decarboxylated to pyruvate, which was oxidized slowly and thus accumulated. The accumulated pyruvate also accepted an amino group from glutamate to form alanine (Altenbern and Housewright, 1953). This pathway of oxidation of glutamate was proposed from oxidative studies with postulated intermediates, the effect of inhibitors, products of oxidation, and the ratio of oxygen uptake to glutamate disappearance (Marr *et al.*, 1953). A tricarboxylic acid cycle is also operative in *B. abortus*, strain 19 (Altenbern and Housewright, 1952). Our result (Dasinger, 1960) with a virulent strain of *B. abortus* (2308) indicates an identical pathway to that found by Marr *et al.* (1953) for a strain of low virulence.

TABLE 7

ROLE OF PERMEABILITY IN GLUTAMATE OXIDATION BY *BRUCELLA ABORTUS*

	Strain	
	11	2308
$QO_2(N)$* (whole cells)	426	115
$QO_2(N)$ (cell-free extract)	35	42
Km (cell-free extract)	1.5×10^{-3} M	1.2×10^{-3} M
Maximum rate of uptake (apparent) μmole/2 min./G. cells (wet wt.)	6.2	2.8

* μL O_2/hr./mg. N (endogenous subtracted).

TABLE 7 is a summary of results from various experiments that illustrates that permeability plays a prominent role in determining the rate of glutamate oxidation by strains of *B. abortus* (Dasinger, 1960). *B. abortus*, strain 11, has a very low virulence for the guinea pig, because 10^7 cells do not produce progressive infection, while *B. abortus*, strain 2308, has an ID_{50} of about 20 cells for production of progressive infection. When we compare the oxidative rates of whole cells with those of cell-free extracts, it is observed that an equalization of oxidative rate occurs with the two strains when the permeability barrier is destroyed by treatment in a sonic oscillator. This treatment also destroys enzyme activity, but we assume this destruction is equal since the cells were treated identically. These results provide indirect evidence in support of permeability as the rate limitation in glutamate oxidation. Direct evidence was obtained, however, with permeability studies. These experiments involved the determination of the initial rates of glutamate uptake by whole cells at different glutamate concentrations. The quantity of glutamate taken up in relation to concentration followed the Michaelis-Menten relationship, which made it possible to calculate the maximum rates of uptake for the strains under consideration. As may be seen from TABLE 7, the cells of strain 11 have a considerably higher rate of uptake than do cells of strain 2308. When we consider that both strains have the same K_m value for the oxidation of glutamate and the same quantity of this enzyme system ($QO_2[N]$ of cell-free extracts), then we can conclude that strain 11 could more completely saturate this system than could strain 2308. This would result in the higher rate of oxidation by strain 11 than is observed with whole cells. This, then, is evidence that permeability of glutamate is the rate-limiting factor.

Let us now reiterate point 3 of our opening paragraph, that is the virulence of brucellae depends on their ability to multiply intracellularly. For a microorganism to multiply intracellularly, it must be able to cope with the intracellular environment. It has also been observed that, with *in vitro* infections of monocytes with brucellae, the intracellular growth of the parasite had no observable effects on the monocytes, even though many of the cells became completely engorged (Holland and Pickett, 1958; Shepard, 1959). Mitotic division can occur in lightly parasitized cells of bovine origin (Richardson, 1959). These results indicate a degree of noninterference with the metabolism of the host cell by brucellae that are able to multiply intracellularly in normal monocytes. On the other hand, ingestion of rough variants by monocytes results in a more rapid destruction of the monocytes, which, if it happened *in vivo*, would expose the bacteria to the bactericidal action of serum (Braun *et al.* 1958; Freeman *et al.*, 1959).

Evidence has recently been presented (Elberg, 1960; Ralston *et al.*, 1960) that glycine renders the cell wall of *B. melitensis* cells susceptible to lysis by a lysozymelike enzyme of rabbit monocytes. This action is exhibited both *in vitro* with crystalline lysozyme and *in vivo* with infected monocytes. Rough cells are more susceptible than smooth cells, and a virulent strain of *B. melitensis* is more resistant than the less virulent strain *B. melitensis* Rev Is. These authors suggest that virulence may be related to the amount and availability of the lysozyme substrate in the cell wall. Resistance to infection may depend on physiological conditions leading to the accumulation of glycine or similarly acting agents, and of lysozyme-like enzymes.

We have described three biochemical differences between smooth strains of low and high virulence. These are: (1) catalase activity; (2) the effect of capryl alcohol on oxidative activity; and (3) the rate of glutamate uptake and oxidation. We shall discuss the first and third of these differences in relation to intracellular parasitism.

Since many of the features of the disease process in brucellosis are similar to those of tuberculosis, some comparisons at this point are in order. It has been demonstrated (Kato *et al.*, 1959) that, upon the onset of hypersensitivity in tuberculosis of mice, the catalase activity of liver tissue is decreased significantly. It is also relevant that *Mycobacterium tuberculosis* strains of low virulence have decreased or there is no catalase activity (Cohn *et al.*, 1954). The decreased pathogenicity of catalase negative strains is indicated by spontaneous healing of lesions after an initial progression (Vincze *et al.*, 1960). It is not improbable that a sufficient catalase activity is necessary to maintain a favorable environment for growth in tissues. Therefore if catalase is not supplied by the tissues, it might be advantageous for the organism to provide this activity. The effect of brucellosis on the catalase activity of host cells is not known, but since there are many events that seem to lower catalase activity in tissue it is not unlikely that lower tissue catalase activity could be a feature of brucellosis. It has been demonstrated with brucellae that strains of low virulence have a low catalase activity, and the same relationship of catalase to virulence seems to hold in the tubercle organism as well as *Pasteurella pestis* (Rockenmacher, 1949). The relation of catalase activity to intracellular growth should be studied with the brucellae.

The relationship of glutamate uptake and oxidation to virulence may have a similar foundation that postulated for catalase, that is, the maintenance of an intracellular environment favorable for multiplication of the parasite. It is evident from studies on the growth of animal cells in tissue culture that glutamine is an important metabolite for survival and multiplication (Eagle, 1959). Glutamine is used in synthesis of purines and pyrimidines and also serves as the primary source for glutamic acid. The glutamic acid then is used in the synthesis of aspartic acid, proline, and serves as an amino donor in synthesis of several other amino acids as well as being necessary for protein synthesis. It seems apparent then that the rapid removal of glutamate from the internal environment by an intracellular parasite would certainly threaten the ability of the host cell to maintain its metabolic integrity. The possibility of rapid removal of glutamate threatening the disruption of the metabolism of the host cell is increased by the fact that there is a high rate of protein turnover in mammalian cells maintained *in vitro* even in growing cultures (Eagle, 1958). If the metabolic integrity of the host cell is upset, lysis of the host cell might be an end result. This would release the bacterial cells to the inimical environment of the serum. On the other hand, an upsetting of the host cells metabolic integrity might trigger biochemical events that would result in the production of conditions that are unsuitable for growth of the parasite. These events are more likely to be precipitated by the strains of low virulence that rapidly remove glutamate so that it is unavailable for the use by the host cell.

We have discussed here only two biochemical differences between smooth strains of low and of high virulence and, undoubtedly, there are many more differences. However, the factors we discussed could contribute to virulence and should be considered further in *in vivo* experiments.

Since several *in vitro* host-parasite systems have been described that utilize brucellae, and since much information is accumulating on the metabolism of cells in tissue culture, we feel that a tissue culture system will provide a useful model for a biochemical investigation of the intracellular growth of the brucellae.

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SOME ATTRIBUTES OF VIRULENCE IN *SHIGELLA**

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Host-Parasite Relationships in Shigellosis

Shigellosis, or bacillary dysentery, is an acute or chronic intestinal disease seen in the natural state only in man and monkeys. Primarily an incapacitating disease with a low death rate, it is marked by a short incubation period, frequent relapse or reinfection, transient or ineffective immunity, and frequently a prolonged convalescent or inapparent carrier state. Severity of clinical symptoms varies widely, ranging from no more than soft or frequent stools to acute abdominal distress, toxemia, and death in 24 hours. The disease is usually self-limiting and is essentially confined to the intestinal tract, predominantly the colon. Intestinal pathology is manifested as a toxic inflammation of the mucosa, with edema, congestion, plasma cell infiltration, lymphoid hyperplasia and, in the more severe cases, ulceration and hemorrhage. Regeneration of mucosal structure may be complete, or a chronic ulcerative colitis with strictures and fibrosis of the muscularis may develop. The infectious process commonly is at a peak in one week and has subsided by three weeks.[1-3]

Shigellosis is caused by any of the organisms grouped in the genus *Shigella*, a nonmotile division of the large and complexly interrelated family of Gram-negative, nonsporing rods, the Enterobacteriaceae.[4,5] Bensted has summarized the principal historical events relating this genus to bacillary dysentery[6] culminating in the recent international classification of its members in four taxonomic subgroups composed of *S. sonnei* and of serotypes related respectively to *S. dysenteriae*, *S. flexneri*, and *S. boydii*.[7] The modern Soviet literature utilizes a somewhat different nomenclature adopted in 1952. Papers by Ewing *et al.*,[8] and Novgorodskaia[9] compare the two classification systems.

Pathogenicity is not used as a criterion in the classification of *Shigella*. However, as Weil has said,[10] certainly it is implied, for pathogenicity for the human intestinal tract is the essential quality justifying the separation of this group of organisms from the many closely related bacilli. Nevertheless, it may well be questioned whether the usual state of shigellae in the natural host is not one of silent or at least inapparent parasitism: Watt states that a clinical case commonly can be shown to be a focus for 8 to 10 culturally positive but completely asymptomatic individuals and that surveys of the general population in lower income brackets have demonstrated as high as 20 per cent of the individuals to be infected.[11] Felson believes, however, that careful sigmoidoscopic examination of such persons will reveal some degree of mucosal damage;[12] this also is the conclusion of Dzhikidze in his extensive study of the dysenteric carrier state in monkeys.[13] However, if McDermott's subdivision of inappar-

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ent infections into dormant and into latent infections is adopted, and also his definition of the latter as a condition in which the presence of the microbe can be detected only when a change in its environment results in overt disease,[14] then unrecognized infection with *Shigella* may well be far higher than is presently appreciated.

Pathogenicity has been defined as a term applying to the disease-producing potential of a class or group of organisms, whereas virulence is reserved to describe the pathogenic capacity of a homogenous strain of an organism observed in a homogenous host population under defined conditions. Implicit is the thought that the experimental system simulates the natural infection, permitting analogies to be made.[15] If we are to be limited in any substantial degree by these criteria, so-called virulence in *Shigella* can hardly be discussed. *Shigella* strains in the laboratory are unstable and nonhomogenous, morphologically and antigenically. In no experimental host does the infection resemble in most essentials that seen naturally in man and monkeys. Only in the experimental mouse has any serious attempt been made to meet the criterion of host homogeneity, but this animal is normally resistant to *Shigella* infection *per os* and requires 10^8 organisms or more for parenteral infection in the absence of a potentiating adjuvant. However, it may be reflected that similar objections apply to a surprising degree to many of our experimental virulence assay systems, whatever the human pathogen.

Ecological Factors Affecting Virulence: Antagonisms and Antibiosis

Any search for virulence factors must take into account the ecology of the pathogen in its natural habitat. With *Shigella* this habitat is normally the lower intestinal tract of man and monkeys. Shigellae are not invasive; normally they may be recovered only from the intestinal wall or lumen, or from immediately adjacent mesenteric glands. In this environment they do not have to contend with many of the host's systemic defenses, but they must compete for a foothold with a large variety of other organisms, the normal microflora of the intestinal tract.

It has been demonstrated with *Escherichia coli* that marked fluctuations occur among strains resident in the gut, but little success has followed attempts to implant competing strains.[16,17] This may be contrasted with Schaedler and Dubos' current report of successful transfer of *E. coli* resident in one mouse strain by feeding it to another that had been raised free of this pathogen.[18] Transfer of laboratory strains, however, was unsuccessful. The work of these investigators, and studies of others[19] on the increased susceptibility of germfree animals to infective agents including *Shigella*,[20] have indicated that the presence of certain commensal bacteria in the intestinal tract also has a direct effect in preparing the host's defensive mechanisms against subsequent infection.

E. coli grown in mixed culture with *Shigella* tends to suppress the latter. This activity has been attributed to the production by certain *E. coli* strains of antibiotic substances known as colicines.[21] Colicines are diffusible substances produced by a number of enteric species including *Shigella*, which show a type specificity in their activity against other enteric organisms. They occur in a substantial proportion of *E. coli* strains isolated from normal persons[22] and in a a much higher percentage of isolations of *E. coli* from *Shigella*-infected persons or their family contacts.[23] Colicinogenic properties may be transferred to other enteric organisms, including different species, by growth in mixed culture. Such transfer is genetically controlled and requires cell conjugation. It is not phage-mediated.[21,24]

Controlled studies *in vitro* and *in vivo* on the role of colicines have indicated, first, that suppression of *Shigella* by *E. coli* is not dependent upon colicine production;[25-27] and, second, that an antibiotically active *E. coli* strain effective in suppressing *Shigella* when both were inoculated in mice intraperitoneally was unsuccessful in protecting against enteric infection by a colicine-sensitive pathogenic *E. coli* strain.[28] These findings imply that, although colicine production may well be an important factor affecting the successful implantation of an enteric organism, other antagonistic mechanisms as yet unknown are also involved.

Bacteriophages active against *Shigella* have been recovered from as many as 50 per cent of stool specimens in a dysenteric outbreak, but their function in the infectious process is not clear.[29] Goodgal has demonstrated that P^{32}-tagged DNA of phage becomes incorporated in the bacterial DNA of *S. dysenteriae*,[30] and others have shown that transduction can alter agglutinative antigens[31] or the capacity to ferment lactose[32] and that such genetic transfer can also take place between *E. coli* and shigellae.[33] Luria and Burrous showed that genetic transfer between these organisms also results from direct mating, the hybrids possessing characteristics from both parent species.[34]

Taken together, the evidence of competitive bacterial antagonisms and of genetically-controlled alteration of the characteristics by which closely related enteric species are differentiated gives a broad hint that in this mosaic of bacterial interactions the key to virulence in *Shigella* should be sought.

Biochemical and Morphological Properties Associated with Virulence

Traditionally the pathogenic properties of the Gram-negative organisms have been explored by determination of the physiological disturbances created by their endotoxins.[35,36] These effects include vasomotor disturbances, hyperglycemia followed by hypoglycemia, fever, leukopenia followed by leukocytosis, tolerance after repeated small doses, and generalized Schwartzman sensitivity. Important recent contributions include those by Benacerraf and his colleagues on the role of the reticuloendothelial system in detoxification of endotoxin.[37] Landy and Shear have demonstrated the presence in mammalian tissues of polysaccharides capable, after enzymatic separation from other cellular constituents, of eliciting host responses strikingly similar to those evoked by endotoxins.[38,39] Dubos and Schaedler have examined the interrelation between nutritional deficiencies and endotoxin in altering the susceptibility of mice to bacterial infection and have demonstrated a synergistic effect that decreases the host's ability to resist toxicity.[40] Fine and his associates have summarized their extensive studies on traumatic shock and present an impressive argument that direct absorption of bacterial endotoxin from the gut under certain conditions provokes vascular collapse and irreversible shock.[41]

These observations may all be pertinent to understanding the pathogenesis of shigellosis; however, similar effects are obtained with endotoxins derived either from pathogenic or nonpathogenic organisms. Accordingly, endotoxins as such cannot be the prime determinants of virulence.

S. dysenteriae differs from other *Shigella* in that both its rough and smooth strains also contain an exotoxin, commonly called a neurotoxin because it produces neurological lesions in mice and rabbits. It also produces edema and hemorrhages in the rat intestine and, with high doses, in rabbits.[42] The rate of spread of neurotoxin after injection in mice or rabbits by various routes, including directly into the sciatic nerve and into the medulla oblongata, gives no evidence of transport through nervous tissue. Rather, the evidence favors dissemination by the blood stream, the lesions being directly attributable to damage to vascular endothelium.[43]

However, neurological lesions or symptoms are uncommon in human dysentery, and the importance of the exotoxin in such infections is not clear: Van Heyningen considers that its pathogenic effects are separate from those of the endotoxin, since specific antitoxin produces a transient clinical improvement although it has no antiendotoxic or antibacterial effect. Also, exotoxin has no aggressive activity that would aid in establishing an infection;[44] like endotoxin, it must be considered to play only a secondary role in virulence.

Little work has been done to relate enzymatic activity in shigellae to virulence. Formal and Lowenthal surveyed the mucinolytic activity of *Shigella* and found mucinases only in three *S. flexneri* serotypes; all other serotypes and members of the other subgroups were negative. They concluded that this enzyme does not play a major role in *Shigella* infection.[45]

Berry and Mitchell, working with *Salmonella typhimurium*, showed in 1953 that administration of intermediates and inhibitors of the tricarboxylic acid (TCA) cycle to mice increased susceptibility to that infection.[46] The occurrence of a TCA cycle in *S. flexneri* was demonstrated by Pan *et al.*,[47] and shortly thereafter Floyd and Clark reported data indicating that TCA cycle intermediates and inhibitors do in fact have an infection-promoting effect on *S. flexneri* 3 infections in the mouse.[48]

With respect to other possibly active enzymes, decarboxylases for histidine, lysine, arginine, and ornithine, but not for methionine, threonine, α-aminobutyric acid, norleucine, or taurine, have been demonstrated in two *S. flexneri* serotypes. It has been suggested that one form of chronic dysentery may be associated with absorption of toxic amines resulting from such metabolic activities, but I am unaware of any experimental evidence in support of this hypothesis.[49]

Morphological variation from smooth to rough in Gram-negative organisms has long been related to loss of virulence for experimental animals.[50] This is attributed to loss of a somatic antigen, a lipopolysaccharide protein complex that is generally found in smooth but not in rough organisms. That smoothness is equally important in the human enteric infection was demonstrated in 1942 by Roelke.[51] Using freshly isolated, morphologically smooth and rough strains of *S. sonnei* recovered from the stools of a single laboratory worker who was infected with a smooth strain, Roelke was able to produce the disease in 7 persons by feeding them 1 to 3 billion organisms of the smooth isolate. The rough strains similarly fed to 18 persons over a period of time produced disease in only one person, from the stools of whom, however, both smooth and rough colonies were obtained.

Colonial color and microscopic appearance under oblique transmitted light have been related to parenteral virulence for mice by Cooper and his associates.[52] These investigators reported that in two *S. flexneri* strains virulent colonies under oblique illumination were red-gold or green-gold and showed various degrees of granularity, crosshatching of the surface, and an internal reflective brilliance that I should like to compare to the internal reflections in an opal. Colonies of low virulence were opaque, relatively nongranular, and pale or pinkish blue.

At our laboratory several of Cooper's criteria were applied to approximately 40 clones isolated from 16 strains representative of all four *Shigella* subgroups.[53] The objective was to determine the precipitin patterns of paired virulent and avirulent clones in an Ouchterlony double-diffusion agar gel system.[54a,b] In evaluating the reliability of using morphological criteria to predict the virulence of a colony, a virulent colony was arbitrarily termed as one containing organisms that, after passage in broth, possessed an LD_{50} for mice of less than 500 cells.

Table 1

Association of Colonial Morphology, Color, and Translucence with Virulence for Mice*

Colonial appearance	Morphology†		Color‡			Translucence‡	
	S	R	RG	GG	BP	Yes	No
Number virulent	18	0	5	5	1	16	1
Number avirulent	8	22	7	1	5	0	11
Correct prediction§ % smooth colonies	69	100	42	83	88	100	92

* LD_{50} determined by intraperitoneal inoculation with mucin.
† Morphologically rough or smooth colonies.
‡ Smooth colonies only; RG, red-gold; GG, green-gold; BP, blue-pink.
§ Data for each colonial characteristic were collected separately.

Using this figure as a break point, it was found that under incident light colonial roughness was associated with avirulence, but 8 of 26 smooth colonies were also avirulent (Table 1). Among smooth colonies viewed under oblique transmitted light, color was a less reliable indicator of virulence than was translucence: blue-pink colonies were predominately avirulent and green-gold virulent; however, red-gold colonies showed no correlation. When selection was made solely on the basis of presence or absence of translucence (that is, internal reflected brilliance), 16 virulent and 12 avirulent predictions were made correctly with but a single error.

Figure 1 shows 5 *flexneri* colonies viewed by incident or reflected light. The 3 colonies in the top row are all highly virulent for mice, with LD_{50} of less than 50 organisms; the 2 in the bottom row are relatively avirulent with LD_{50} in excess of 1000 organisms.

FIGURE 2 shows similar colonies viewed by transmitted oblique illumination. In the top row, from left to right, are translucent colonies with the green-gold, red-gold, and red-gold with surface crosshatching characteristics; the two below possess the dull opacity and pinkish-blue color.

Cooper's[64] blue-pink avirulent colonies were rough on the basis of agglutination in neutral 1:500 acriflavine solution, whereas his virulent colonies were smooth. In contrast to this, it was found that all five of these clones, including the one that was morphologically rough (FIGURE 1), were smooth by the acriflavine test. Cooper also found that his virulent red-gold and green-gold colonies from a single strain were antigenically identical as judged by agglutination and agglutinin absorption tests. This was not the experience with the clones from which the three virulent colonies shown in the top row of FIGURE 2 were obtained. Two clones gave identical cross agglutination titers, whereas the third (FIGURE 2*b*) gave lower titers (TABLE 2). However, all three showed marked nonidentity in terms of precipitin patterns formed in an Ouchterlony agar gel system.

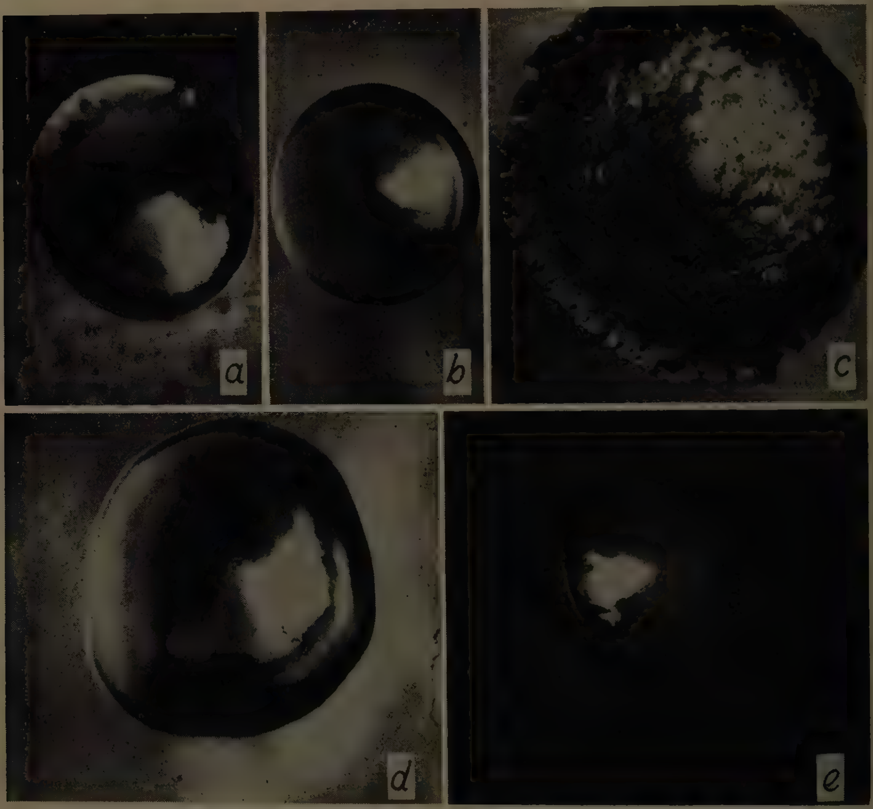

FIGURE 1. Virulent (*a*, *b*, *c*) and avirulent (*d*, *e*) *Shigella flexneri* colonies illuminated by incident or reflected light.

FIGURE 3 shows the reaction of high titer, concentrated rabbit antisera prepared against each of the three virulent clones diffusing outward from the central cup in each plate against sonic lysates prepared from each of the five virulent and avirulent clones and from the original strain. I shall attempt no detailed description for it is evident that the reaction patterns are quite dis-

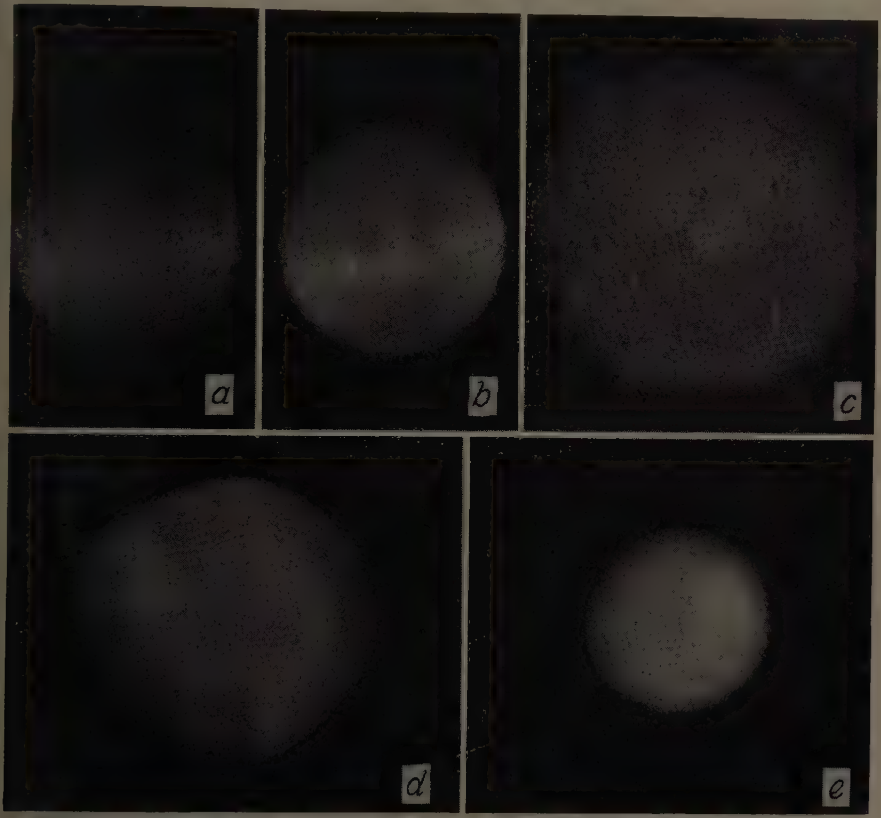

FIGURE 2. Virulent (*a*, *b*, *c*) and avirulent (*d*, *e*) *Shigella flexneri* colonies illuminated by oblique transmitted light.

TABLE 2

CROSS-AGGLUTINATION OF THREE VIRULENT *SHIGELLA FLEXNERI* CLONES ISOLATED FROM A SINGLE STRAIN*

Antigens	Antisera		
	I	II	III
I	5120†	5120	5120
II	640	1280	640
III	5120	5120	5120

* See colonies, FIGURE 2*a*, *b*, *c*, and text.
† Reciprocal of titer.

similar. This is not to be attributed to differences in reactivity of individual rabbits during immunization, since the precipitation patterns depicted in FIGURE 3 are each illustrative of reactions obtained with antisera from 4 to 6 animals.

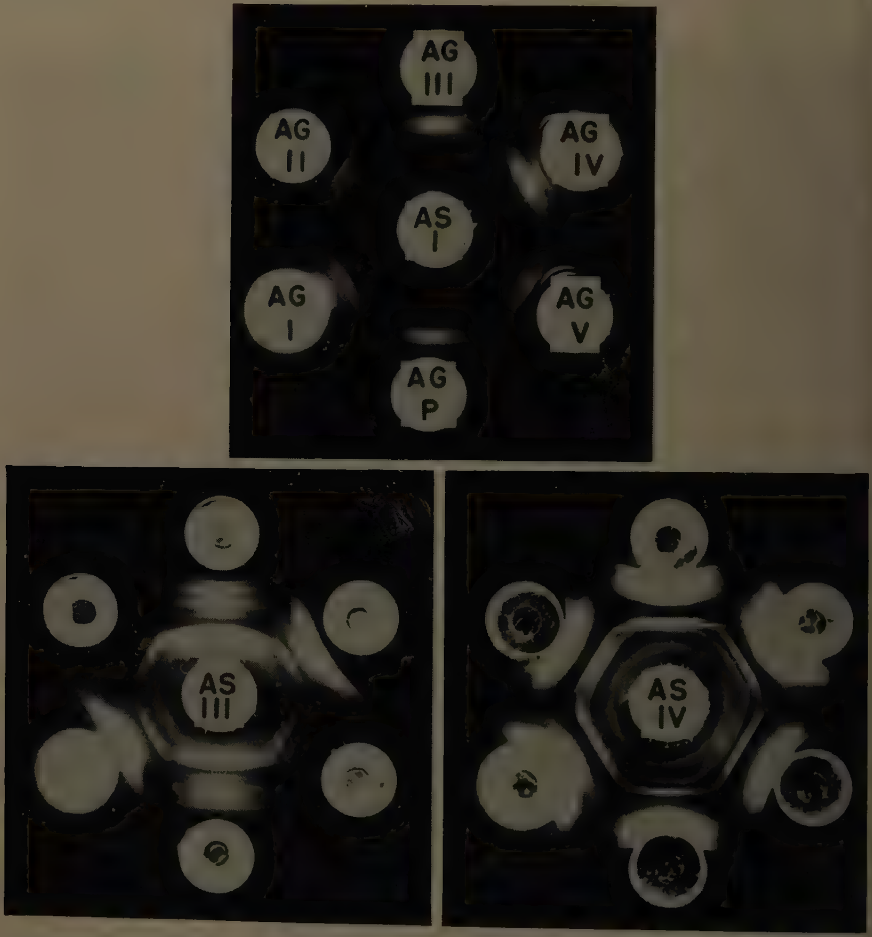

FIGURE 3. Ouchterlony gel precipitation patterns of antisera prepared from three virulent *Shigella flexneri* clones isolated from a single strain.

It is obvious that such diversity among patterns from virulent clones makes it hazardous to offer any opinion concerning an association between precipitation pattern and virulence. Nevertheless, I must note that on several occasions an apparent qualitative or quantitative loss of a precipitin line in the reaction of the avirulent member of paired clones has been observed. FIGURE 4 presents an example of this in the reactions of two *S. sonnei* antigen preparations (AV and V) in the figure, reacting against antiserum (AS) to the parent strain in the central cup. Clone (AV) is avirulent, clone (V) is virulent; the former lacks a precipitating antigen possessed by the latter. However, in view of the complexity of antigenic relationships even within a single *Shigella* strain and the number of variables that are now known to affect the precipitin patterns of these organisms, I do not believe any specific biological significance can be attributed to such observations at this time.

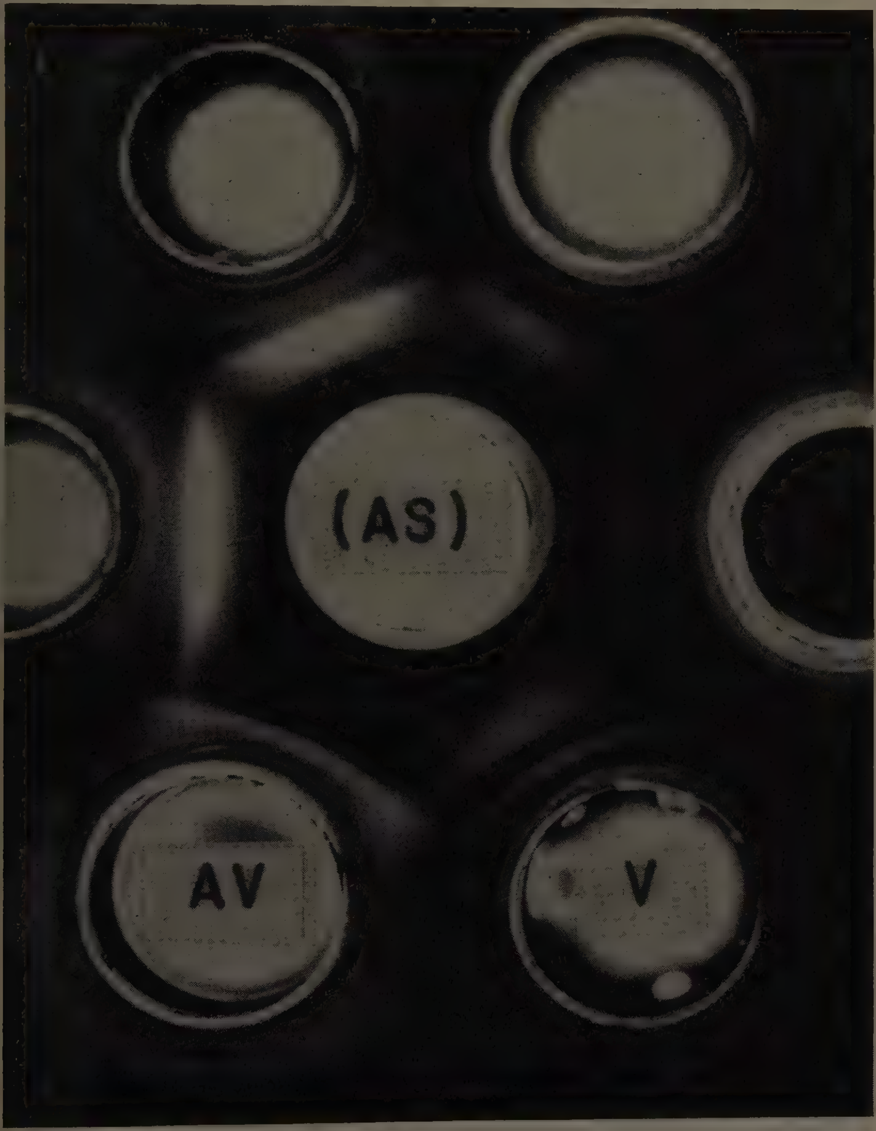

FIGURE 4. Ouchterlony gel precipitation patterns of a virulent and an avirulent *Shigella sonnei* clone isolated from a single strain. AV, avirulent clone; V, virulent clone; AS, antiserum to parent strain.

Recently a new morphological structure was found by electron microscopy in both recently isolated and older strains of certain *flexneri* serotypes that may prove to have some relationship to cellular invasiveness.[55] These serotypes possessed nonflagellar filamentous appendages termed "fimbriae," which can be produced by selective serial cultivation in broth. Fimbriate bacilli show hemagglutinating activity and were observed to adhere to epithelial cells from the guinea pig and human colon. Nonfimbriate organisms had neither of these properties.

In summary, several morphologic and biochemical properties are known that are associated with experimental parenteral virulence in laboratory animals, and the experimental production of many of the pathologic and pharmacological signs of the enteric disease is quite feasible. There is good evidence that one colonial morphologic characteristic—smoothness—is associated with disease potential for man. However, information is lacking concerning the contribution of such factors to colonization and onset of infection in the intestinal canal of a natural host. This is all the more confusing since most information on virulence has come from experiments with parenteral infections of mice, an infection model that Shaughnessey showed had little relation to the disease in man.[56] Working with human volunteers in 1946, he found in *flexneri* strains no correlation between parenteral virulence for mice and capacity to produce clinical infection in the human.

Virulence and Pathogenesis of Shigella in Experimental Infection Models

In the past 20 years the search for a suitable laboratory model for *Shigella* infections has been much intensified. Workers in the Union of Soviet Socialist Republics in particular have explored many possibilities. One recent article in Soviet literature reported that infection was attempted in representatives of all classes ranging from coelenterates through arthropods, amphibians, and mammals, including 18 species of the last.[57] Only mammals became infected, and in these it was apparently necessary to administer doses *per os* in excess of 10 billion organisms. On the basis of the clinical picture, the authors recommend macaques as the animal of choice, second place going to young bears, with the kitten as third choice. Others also have used kittens as a host.[58,59]

In their very extensive vaccine program, most Soviet workers utilize the laboratory mouse inoculated parenterally without adjuvant. A satisfactory LD_{90} is stated not to exceed 500 million organisms.[60] This must be contrasted with the practice in this country in similar work, since the early 1940s, of adding mucin to the inoculum as an infection-promoting substance, with resultant LD_{50} for strains highly virulent for mice of less than 10 organisms. The infection in mice in both cases, however, shows little if any resemblance to that seen in man, since massive invasion of blood stream and organs takes place.

Recently attempts have been made to produce an enteric infection in mice. E. E. Baker in 1953 (personal communication) and D. M. Eisler in our laboratory in 1958 (unpublished observations) attempted by gavage to infect with *S. flexneri* a total of 23 inbred strains of mice, utilizing various conditions of stress. The results were essentially negative, although transient bacteremias and sporadic deaths were recorded. McGuire and Floyd also reported bacteremia and minor pathological changes in the viscera of albino mice infected orally, per rectum, or intraperitoneally.[61] The LD_{50} however was approximately 5 billion organisms, an enormous dose, although it was at least one log lower than the toxic dose with heat-killed organisms. Fasting the animals did not lower the LD_{50}.[62]

Freter has approached the problem of inducing enteric infections in mice by means of prior suppression of resident bacterial antagonists.[63] This was accomplished by administering streptomycin followed by challenge with a streptomycin-resistant *S. flexneri* strain. The resultant colonization of the experimental host's intestinal tract produced no clinical disease but maintained an infection as evidenced by the shedding of organisms in the feces throughout the two-week observation period. Simultaneous inoculation of a noncolicine-producing *E. coli* strain eliminated all *Shigella* within 48 hours.

Cooper extended and confirmed Freter's work, showing that infection could be induced with 100 organisms and occasionally with as few as 15.[64] He also reported on gross and microscopic findings in his mice, noting lesions that he interpreted as similar to those seen in the chronic human disease. In addition, he noted that antibiotics alone produced size increases in the mouse cecum and appendix, indicating a change in the normal physiology of the host.

Formal and his associates in a series of papers have described the development of a fatal infection model in guinea pigs.[65-67] They obtained lethal infections by starving their animals for 4 days or by injecting carbon tetrachloride subcutaneously, followed by oral calcium carbonate prior to administration of organisms by means of a stomach tube. Opium was also given intraperitoneally. Evidence was presented that the pathogenesis was dependent upon liver damage that did not affect removal of toxic substances from the blood but that did interfere with their detoxification.

These studies on mice and guinea pigs indicate that, with sufficient manipulation of the host, experimental *Shigella* infections can be induced. They offer additional evidence on the role of the normal microflora in preventing initial colonization by *Shigella* and on the role of toxic damage in the subsequent pathogenesis of the experimental disease. However, their value as assay systems for virulence is limited in the mouse model by the failure to produce an acute disease and in the guinea pig model by marked irregularity of response of such strenuously pretreated animals to graded *Shigella* dosage.

Several groups of workers in Europe have studied the possibility of using as a dysentery infection model the reaction of the guinea pig or rabbit conjunctiva.[68-74] Following inoculation with *Shigella*, in 24 hours acute purulent conjunctivitis develops that progresses to corneal opacity and ulceration after 48 hours. The process regresses and the purulent discharge diminishes gradually, the infected eye becoming normal after 3 weeks to 3 months. The infection is diagnostic only of freshly isolated strains of *Shigella*, since old laboratory strains and other members of the Enterobacteriaceae fail to produce a reaction. One exception was reported by Siroko, who stated that strains of *Salmonella heidelberg* also produced a conjunctivitis that, however, could be differentiated clinically by a longer incubation period (3 days) and complete regression after 3 to 4 days of infection.[75] Siroko also recommended this method as a means of eliminating so-called atypical dysentery strains that in fact are not pathogenic and do not belong with the dysentery group.

There is some disagreement among these investigators as to the extent and nature of the systemic and local immunity that develops. Sereny, who has worked most extensively with this system, makes a good case that the disagreement results from differences in technique.[76] He considers that the infection confers both clinical and humoral immunity, but in a degree too low to prevent massive reinfection. Humoral immunity confers protection on the uninfected eye and is specific to the infecting organism. Tissue immunity develops in the infected eye and is of a higher degree and nonspecific. Sereny believes these features are analogous to those seen during convalescence in human dysentery.

The use of monkeys as experimental hosts in dysentery would seem offhand to be entirely feasible, but successful experimental infection of this host has proved to be most erratic. There appears to have been little study of experimental dysentery in this animal until the late 1930s. Then Preston and Clark reported that the acute disease in this host was comparable clinically and pathologically to that in man.[77] Janota and Dack[78] and later other workers[79] commented on the fact that vitamin-deficient diets frequently precipitated acute dysenteric attacks. This was shown primarily to be due to folic acid deficiency although a lack of vitamins has also been implicated.[80,81] Hoskins and Dack, in an attempt to implant a known *flexneri* strain in 10 "vitamin M"-deficient monkeys, succeeded in only one, although two others became acutely ill after a second challenge.[82] Branham was unable to infect monkeys orally with large doses of *S. dysenteriae*.[83] Most of these workers used enormous doses, from 1 to 10 billion or more organisms. Objecting to such dosage, Forsyth in India successfully infected his animals by feeding them bananas impregnated with mucus from a dysenteric monkey,[84] this being the first instance of which I am aware in which doses successfully used could be considered comparable to the contamination received by caged monkeys who so readily are infected by their cage mates. Recently Floyd and McGuire in studies with large numbers of monkeys have confirmed that direct transfer of organisms between caged animals produced significantly higher percentages of clinical infections than occurs when the organisms are passed on laboratory media.[85] However, they reported that even under these conditions about 30 per cent of the animals failed to become infected.

In the late 1940s, a systematic study was begun at the Sukhumi Institute in the Union of Soviet Socialist Republics on a large herd of macaques known to be carriers of *S. flexneri*; these studies have continued to the present time and have recently been summarized by Troitzky[86] and by Lapin.[87]

Finding that they were unable to infect their carrier monkeys with *flexneri* strains but that they could do so successfully with *S. sonnei* that was not prevalent in the colony, workers at this laboratory undertook a program of careful bacteriologic examinations of each animal in the colony, instituting chemotherapeutic treatment of all carriers when discovered. This was later supplemented by combined peroral and subcutaneous vaccination. They cite figures to illustrate that both the carrier rate and the number of positive recoveries of *Shigella* decreased progressively as this regimen was used over a period of several years. As the carrier rate decreased they found themselves able to infect members of the herd with *flexneri* strains under the same conditions that had formerly failed.

These are extensive studies, and only their pertinent conclusions can be mentioned here. The carrier state provides a low-grade infection immunity that can protect the host against a provocative challenge only if it is continuously reinforced by repeated low-grade exposures to the same organism. Post-infection immunity exists, but is also of low-grade and short duration, lasting only a few weeks. It is subgroup-specific, but evidence is not available as to whether it is type-specific.

In either case the immunity is readily overwhelmed to produce an acute infection or exacerbation. This can be caused by an overload with new organisms, by specific allergic reaction such as often follows administration of an oral vaccine to an animal still shedding organisms, or it can be caused by completely heterologous factors, such as measles, pregnancy, hypovitaminoses, or sublethal X irradiation. The key factor in changing a benign, completely inapparent infection into active disease is thought to be any stress capable of increasing the permeability of the intestinal wall.

In our laboratory, work has also been done with dysentery infections in monkeys (unpublished observations). Initially an unsuccessful attempt was made to induce an acute dysentery attack in a group of 6 *Macacus irus* monkeys by oral administration, repeated at 4 to 6 week intervals, of 3 highly mouse-virulent *S. flexneri* strains. These attempts seemingly produced no clinical effect and no shedding of organisms beyond a few days, although serum agglutinins developed. A second series of 9 animals was then subjected to serial oral challenge with a single or with a pool of several *S. flexneri* 3 strains of monkey origin. Briefly, all 9 animals gave some degree of clinical response, ranging from apparent malaise and anorexia, to death from acute hemorrhagic dysentery accompanied by marked inflammation, hemorrhage and superficial mucosal ulceration of the colon and distal ileum. However, the noteworthy point is that all these responses occurred after, and only after, the second challenge. As many as 4 subsequent challenges with the same strain at 10- to 30-day intervals had no effect on the survivors. However, after a 6-month rest period, in 3 of 4 survivors the same process was successfully repeated: namely, a challenge without effect followed after 6 weeks by a second challenge that produced acute illness.

The implication is clear that enhanced virulence resulted from prior sensitization of the host. Goldgraber and Kirsner have reviewed the experimental evidence that the gastrointestinal tract, including the colon, can participate in hypersensitivity reactions.[88] Mokhnach has advanced the hypothesis that dysentery pathogenesis is dependent upon specific sensitization of reticulo-endothelial elements of the large intestine to bacterial endotoxins, disease resulting from specific or nonspecific factors that increase the permeability of vascular endothelium or of mucosal epithelium to permit the sensitized reticulo-endothelial cells to receive an increased provocative allergin dose.[89]

The role of hypersensitivity in experimental dysentery has not been systematically investigated, although it would appear that a suitable procedure is now available for this purpose by the technique of infecting ligated rabbit gut loops. This procedure has been successfully used by Taylor *et al.* to study pathogenic *E. coli*,[90] and by Jenkin and Rowley to evaluate factors involved in the pathogenesis of experimental cholera.[91] Injection of a pathogenic strain into the ligated gut produces distension from edema and exudate and inflammation and desquamation of the mucosal epithelium; the presence of living organisms is required to produce these responses. Jenkin and Rowley note that positive loops result from injection of organisms capable of multiplication to high levels; negative loops show little or no multiplication of the injected strain.

In our laboratory an analogous relationship between the capacity of a *Shigella* strain to multiply to high levels intracellularly in tissue culture and the ability of that strain to produce disease when fed to monkeys sensitized as described above has been demonstrated (unpublished observations).

S. flexneri and *S. sonnei* strains were grown extracellularly or intracellularly in chick fibroblast or in Henle intestinal epithelial cell tissue cultures.[92] When growth was extracellular, peak yields were obtained only in the presence of living tissue cells and represented at least a 100-fold increase over counts that could be obtained in tissue-free controls or in the presence of heat-killed tissue cells. Intracellular infection was obtained in the manner described by Shepard,[93] by incubating the tissue monolayer in an infection medium containing inactivated normal horse serum to promote uptake of the bacteria by the tissue cells. Extracellular growth of organisms was controlled by changing the medium and washing the monolayer at 2- to 3-hour intervals.

Plate counts of viable bacteria released from tissue monolayers disintegrated by trypsinization and shaking with glass beads provided data on intracellular growth (unpublished data). FIGURE 5 shows growth curves typical of the 9 *flexneri* and *sonnei* strains studied; it is apparent that the strains show two different growth patterns determined by the individual strain's capacity to survive and to initiate multiplication during the early period of intracellular residence. Since the subsequent curve of multiplication for all strains was essentially the same, peak yields in the two groups differed by 1 to 2 logs. The same strains grown in heart infusion broth show no such difference, as is also shown in FIGURE 5.

Although direct evidence is lacking, it may be speculated that the capacity of a *Shigella* strain to produce high yields when grown in intimate association with living cells is a requisite for virulence. Taylor *et al.* showed in their rabbit experiments that pathogenic *E. coli* adhere in a layer on the columnar epithelium of the villi,[90] and Jenkin and Rowley demonstrated that pathogenic *E. coli* and *Vibrio cholerae* strains differ from avirulent strains in their ability to produce markedly higher counts in the lumen of the ligated rabbit gut.[91] Schaedler and Dubos[94] have also noted the firm adherence to the intestinal wall of strains of *E. coli* freshly isolated from normal mice and capable of colonizing the intestinal tract of "specific pathogen-free" mice, whereas old laboratory strains that could not colonize showed no adherence.[18] Piechaud and his coworkers, in a detailed histological description of experimental *Shigella* infections in the guinea pig conjunctiva, published photographs of the infecting organism in the cytoplasm of epithelial cells.[94] Finally, in our laboratory we have demonstrated an association in *flexneri* strains between virulence for monkeys and a capacity for high intracellular yields (TABLE 3).

Two strains that gave high yields in intestinal epithelial cell tissue cultures, when grown in broth and fed to groups of 4 monkeys in high dosage (approximately 10^{10} organisms) as previously described, produced moderate to severe clinical dysentery. Under similar conditions, 2 other *flexneri* strains and 1

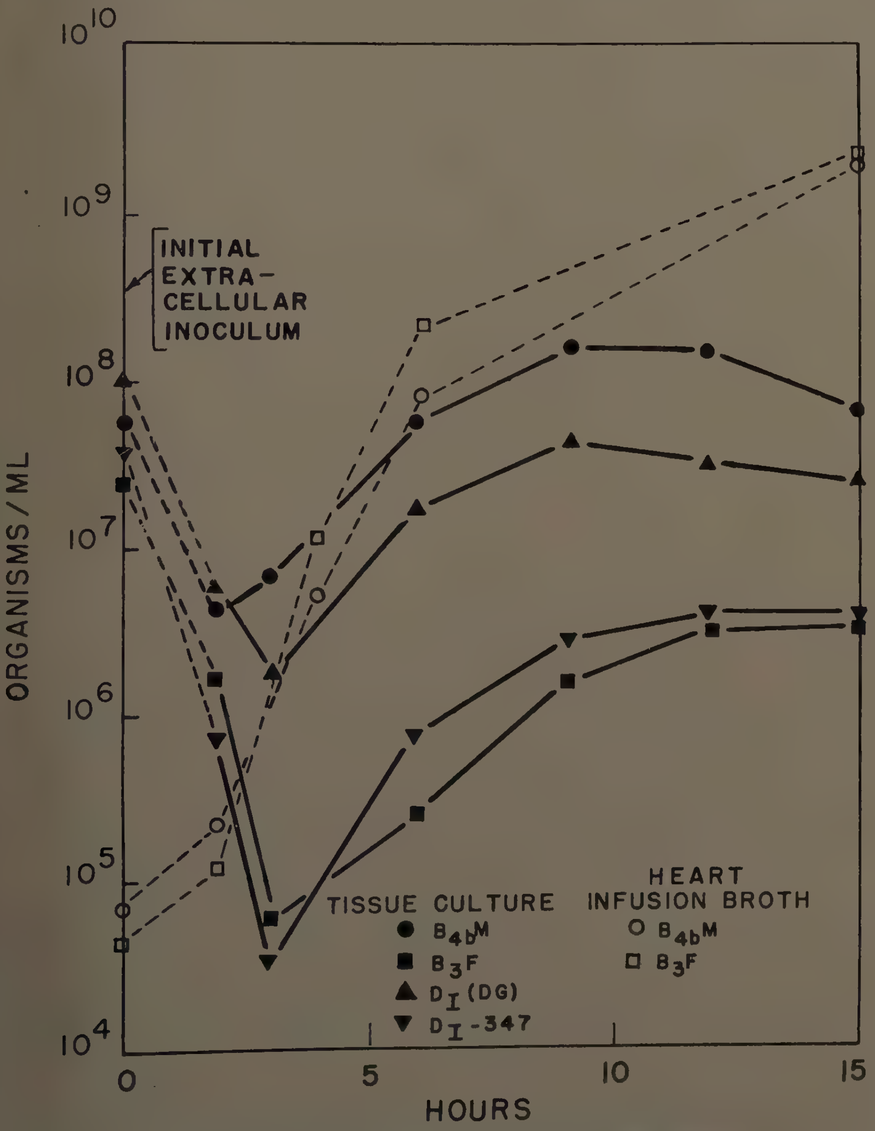

FIGURE 5. Growth of shigellae in tissue culture and in heart infusion broth.

sonnei strain that gave relatively low intracellular yields and that were markedly virulent for mice on parenteral inoculation with mucin, had no effect when fed to monkeys.

Accordingly, it appears that experimental dysentery in the monkey is subject to great variation in the response of individual animals, dependent in some measure on the status of previous exposure to the infecting organism that may have produced a carrier state, low-grade immunity, or tissue sensitization. Virulence in *Shigella* strains has been associated with their growth response to living tissue cells, but what this response implies in terms of biochemical or enzymatic properties of the strain is unknown. Factors that permit natural infection with small numbers of organisms are also unknown, but evidence from other infection models indicates that preconditioning of host tissues by a variety of stress factors, preclusive competition, and perhaps genetic interchange with resident microflora, may be involved.

TABLE 3

VIRULENCE ASSOCIATED WITH INTRACELLULAR GROWTH OF *SHIGELLA* STRAINS

Strain	Intracellular growth*	Virulence†	
		Mouse (I.P.)	Monkey (oral)
flexneri			
B$_4$bM	4×10^7	600‡	yes
B$_3$M	2×10^7	150	yes
B$_4$aM	1×10^7	>1500	—
sonnei			
D$_1$(DG)	2×10^7	>1500	—
flexneri			
B$_3$F	2×10^6	<10	no
B$_3$I	1×10^6	<10	no
sonnei			
D$_1$-347	8×10^5	<10	no
flexneri			
B$_1$aII	1×10^6	>1500	—
B$_4$bS	1×10^6	>1500	—

* Organisms grown 6 hours in human intestinal eipthelial cell tissue culture.
† Organisms grown in heart infusion broth.
‡ Organisms/LD$_{50}$.

Conclusions

The shigellae carry out their vital activities, in a sense, on the body surface in a habitat where they must contend with bacterial competitors in an unstable equilibrium under conditions that for the most part have not been defined. Normally this equilibrium is adequate to restrain if not to eliminate their multiplication. Periodically, however, the equilibrium is upset by factors that affect the host, the parasite, the competing organisms, or most likely, all three in a complex interaction. I suggest that it is this interaction that defines virulence and that it is this point in the infectious process that determines what is to follow.

When the equilibrium fails (from the viewpoint of the host) disease follows; this process still appears to be essentially the result of vascular damage from inability to detoxify excessive amounts of endotoxin.

In recent years, substantial progress has been made in developing a number of infection models with which to study these problems. I suggest that they might well be applied, on the one hand, to assessing the role of allergic reactions in this infection and, on the other, to investigating the possibility among closely related organisms of genetic transfer of factors affecting virulence in *Shigella*.

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THE HOST AS A GROWTH MEDIUM*

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The categories of parasitism may be determined by the response of the host to the entry or presence of the parasite. Pathogenicity is distinguished from saprophytism and symbiosis in that, by displaying a disease, the host is adversely affected by the parasite. Under certain conditions, it may be possible for a given relationship to shift from one category to another. Consequently, pathogenicity must always be defined as an expression of a specific, dynamic host-parasite relationship. Such a definition, which would also apply to the other categories of parasitism, is basically ecological and emphasizes the host as an environment for the parasite.

Pathogenicity as it concerns the parasite assumes that the parasite is capable of utilizing the host environment as a growth medium and that it can overcome the total defense mechanisms of the host. However, many other factors in the host and in the parasite have also been implicated in the manifestation of a disease. By applying a broad point of view, it has been possible to assemble these factors into a scheme:

$$\text{entry} \rightarrow \begin{array}{l}\text{multiplication}\\ \text{and metabolism}\end{array} \rightleftharpoons \begin{array}{l}\text{micro-ecological}\\ \text{alterations}\end{array} \rightleftharpoons \begin{array}{l}\text{production of}\\ \text{host-damaging}\\ \text{factors}\end{array} \rightarrow \text{disease}$$

According to this scheme, pathogenicity represents the successful completion of all steps, and a block at any step results in the loss or absence of pathogenicity. A genetic or environmental alteration in the host, the parasite, or both may be responsible for a block.

The host may be resistant if it does not provide a suitable growth medium for the parasite or presents effective defense mechanisms that prevent, inhibit, or restrict the potential proliferation and metabolism of the parasite. These considerations led to the formulation of a nutrition-inhibition hypothesis of pathogenicity that specifically considers the multiplication and metabolism of the parasite after entry (Garber, 1956).

If the nutritional environment in the host is inadequate, the parasite will not be able to proliferate or to metabolize extensively and will not be virulent; if the nutritional environment is adequate, the parasite may proliferate and metabolize and yet not be virulent. In the absence of an effective inhibitory environment, a parasite in an adequate nutritional environment generally will be virulent; in the presence of an effective inhibitory environment, the parasite will not be virulent regardless of the nutritional environment.

The nutritional requirements of the pathogen must be satisfied at the site of inoculation or of localization. If the parasite proliferates and metabolizes extensively after entry, the host is undoubtedly providing all the required nutrilites; a susceptible host *ipso facto* presents an ineffective inhibitory environment. However, the direct relationship between the host as a growth medium and the specific nutritional requirements of the parasite has been subjected to investigation only within the last decade.

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Experimental evidence indicating that the host may or may not be providing specific nutrilites for the parasite has come from studies using nutritionally deficient mutants of animal and plant pathogens, including bacteria and fungi. Since these mutants were isolated after exposure to mutagenic agents, it was necessary to insure by appropriate tests that the only significant alteration in the pathogens was in its nutritional status. For example, Bacon *et al.* (1950) found that 1 of 11 histidine-requiring mutants of *Salmonella typhosa* was avirulent for the mouse; the avirulence of this mutant was not related to its need for histidine.

TABLE 1

VIRULENCE OF NUTRITIONALLY DEFICIENT MUTANTS OF *KLEBSIELLA PNEUMONIAE* FOR MICE*

Mutant	Requirement	Route of inoculation			
		Intraperitoneal		Intranasal	
		Dosage	Dead/total	Dosage	Dead/total
Kp	None	1×10^4	9/10	3×10^5	9/10
34	Threonine	1×10^4	10/10	3×10^5	10/10
2	Tyrosine	1×10^4	9/10	3×10^5	8/10
40	Methionine	1×10^4	9/10	3×10^5	10/10
27	Leucine	1×10^4	9/10	3×10^5	8/10
17	Histidine	1×10^4	10/10	3×10^5	10/10
22	Uracil	3×10^3	4/5	7×10^4	2/5
2711	Guanine	3×10^4	0/10	8×10^5	0/10
58	Adenine	1×10^5	0/10	3×10^6	0/10
58-01	None	1×10^4	9/10	3×10^5	9/10
58-02	None	1×10^4	6/10	3×10^5	9/10

* Reproduced by permission of *Proceedings of the National Academy of Sciences* (Garber *et al.*, 1952).

Three different mechanisms may be responsible for the loss of virulence of nutritionally deficient pathogens (Garber, 1959): (1) the host provides an inadequate concentration of the nutrilite required by the pathogen for proliferation and metabolism; (2) the host provides an adequate concentration, but other compounds may inhibit or prevent the uptake of the required nutrilite; and (3) the host provides an adequate concentration, but other compounds may be needed to facilitate the uptake of the required nutrilite. Examples of each mechanism will be presented.

The virulence of nutritionally deficient mutants of *Klebsiella pneumoniae* was determined by inoculating mice intraperitoneally or intranasally (Garber *et al.*, 1952). Mutants requiring certain amino acids or a pyrimidine, uracil, retained their virulence (TABLE 1). Cultures from the peritoneal fluid of animals succumbing after inoculation by either route were tested to determine if the nutritional requirement was still present. In all cases the recovered cultures were still mutant, indicating that reversion to the parental or prototrophic type had not occurred *in vivo*. Finally, peritoneal fluid was tested and its ability to satisfy the nutritional requirement of the virulent mutants was established. These observations support a direct relationship between the nutritional need of the virulent mutants and the availability of the required nutrilite in the host.

Purine-requiring mutants of *K. pneumoniae* were avirulent for the mouse when inoculated either intraperitoneally or intranasally. Peritoneal fluid did not satisfy the nutritional requirement of the mutants. Prototrophic reversions from a purine-requiring mutant were virulent when inoculated by either route. Finally, the virulence of a purine-requiring mutant was restored by injecting graded amounts of a solution of one purine or a pool of purines into the peritoneal cavity (TABLE 2). Cultures recovered from the peritoneal cavity and lungs of dead mice were avirulent, indicating that reversion *in vivo* to prototrophy had not been responsible for the death of the mice. Moribund mice were sacrificed during the course of the purine injections and increasingly greater numbers of mutant bacteria were isolated.

TABLE 2

THE EFFECT OF INTRAPERITONEAL INOCULATION OF THE "COMPLEX" PURINE-REQUIRING MUTANT, 27139, OF *KLEBSIELLA PNEUMONIAE* AND INTRAPERITONEAL INJECTION OF HYPOXANTHINE OR OF A POOL OF PURINES*

Experiment	Treatment	Total dosage	Dead/total
I	27139	1.3×10^5	0/10
	Hypoxanthine	50 mg.	0/10
	27139 plus Hypoxanthine	1.3×10^5 50 mg.	3/10
II	27139	8×10^6	0/10
	Purine pool	24 mg.	0/10
	27139 plus Purine pool	8×10^6 24 mg.	10/10

* Fifteen mg. hypoxanthine, 3 mg. xanthine, 3 mg. adenine, 3 mg. guanine. Reproduced by permission of *Proceedings of the National Academy of Sciences* (Garber *et al.*, 1952).

Purine-requiring mutants of other animal pathogens have been reported to be avirulent for the mouse: *S. typhosa* (Bacon *et al.*, 1951), *S. typhimurium* (Gowen *et al.*, 1953), *Pasteurella pestis* (Burrows and Bacon, 1954), and *Pseudomonas pseudomallei* (Levine and Maurer, 1958).

Disease-resistant varieties and species of plants are common. Since plants apparently lack the complex defense mechanisms found in animals, it seemed reasonable to investigate the role of nutrition in the virulence of plant pathogens. The same procedures developed for animal pathogens were employed. Furthermore, experiments with plant hosts are not usually complicated by problems of latent infection, intricate relationships among defense mechanisms, or genetic heterogeneity generally encountered in animal hosts.

Erwinia aroideae is a bacterial plant pathogen responsible for a soft rot of the fleshy storage organs of numerous vegetable species. The surface of slices of storage organs from several host species was inoculated with biochemical mutants of this pathogen (TABLE 3). The hosts exhibited a pattern of resistance or susceptibility that was interpreted as an expression of a relationship between the demand for required nutrilites by the parasite and the availability of these nutrilites at the site of inoculation (Garber, 1954). Further experiments using varieties of two host species, turnip and radish, also revealed a pattern of resistance and susceptibility (Garber *et al.*, 1956). It was possible to apply the methods previously developed for mutants of animal pathogens to demonstrate a direct relationship between the loss of virulence and an apparently inadequate nutritional environment in the host for many but not all mutants.

The resistance or susceptibility of 12 plants from three turnip varieties to three histidine-requiring mutants of *E. aroideae* was investigated. Each plant provided three slices for the inoculations; the remainder of the storage organ was used to determine its free histidine content. The data in TABLE 4 indicate that certain plants were resistant to one or more mutants even though the concentration of free histidine was more than adequate for the proliferation of these mutants. However, when the bacteria were suspended in nutrient broth rather than the normal saline solution, the routine procedure, the previously resistant plants were now susceptible. The histidine content of the nutrient broth was negligible compared with the concentration of histidine available to the mutants at the surface of the slice. Finally, plugs of tissue from resistant organs were added to pour plates of mutants in minimal medium. A halo of bacterial growth around each plug indicated that sufficient histidine had diffused from the surface of the plug into the medium to support the proliferation of the mutants. In several cases, the plugs exhibited a soft rot.

TABLE 3

VIRULENCE OF NUTRITIONALLY DEFICIENT MUTANTS OF *ERWINIA AROIDEAE* ADDED TO THE SURFACE OF SLICES OF TISSUE FROM FIVE HOST SPECIES*

Nutritional deficiency	Hosts				
	Cucumber	Potato	Radish	Turnip	Carrot
Methionine	S	S	S	S	V
Arginine	S	S	R	R	V
Isoleucine and valine	S	S	S	R	R
Threonine	S	S	R	R	R
Leucine	S	S	S	V	S
Cysteine	S	V	R	S	R
Histidine	S	S	S	S	R

S, susceptible; R, resistant; V, some individuals susceptible, others resistant.

* Reproduced by permission of *Proceedings of the National Academy of Sciences* (Garber, 1954).

Histidine-requiring mutants of *Neurospora crassa* have provided an explanation for the behavior of mutants of *E. aroideae* requiring this amino acid. Mathieson and Catcheside (1955) reported that the uptake of histidine by this fungus could be completely inhibited if histidine and other unrelated and nonrequired amino acids were present in broad but specific molar ratios. In the case of the bacterial mutants, adding nutrient broth to the surface of the slices may have altered the molar ratios for histidine and the uptake-inhibiting amino acids. The diffusion of histidine and these amino acids from the surface of the tissue into the agar of the pour plate may have altered the molar ratio present in the agar and at the surface of the plugs.

TABLE 4
THE FREE HISTIDINE CONTENT AND RESPONSE OF TWELVE PLANTS OF THE EXTRA-EARLY PURPLE-TOP MILAN VARIETY OF TURNIP TO INOCULATION WITH THREE HISTIDINE-REQUIRING MUTANTS OF *ERWINIA AROIDEAE**

Plant	Histidine (µg.); fresh weight (gm.)	Mutants		
		8-49	9-46	24-26
10	19.2; 20.0	S	R	R
1	27.3; 27.3	R	R	R
6	32.2; 35.6	R	R	R
5	45.4; 45.4	S	R	R
12	64.0; 64.0	S	S	R
4	63.6; 69.2	R	R	R
11	97.3; 101.6	R	S	R
3	107.5; 107.5	S	S	S
2	105.2; 109.5	R	R	R
7	144.2; 145.9	R	R	R
8	Contaminated	R	R	R
9	Contaminated	R	R	R

S, susceptible; R, resistant.
* Reproduced by permission of *American Journal of Botany* (Garber, 1958).

TABLE 5
THE NUMBER OF SUSCEPTIBLE PLANTS (TWELVE PLANTS PER TEST) OF THREE TURNIP VARIETIES INOCULATED WITH HISTIDINE-REQUIRING MUTANTS OF *ERWINIA AROIDEAE* IN 1956 AND 1957*

Mutants	Foliage		Purple top white globe		Extra-early purple-top Milan	
	1956	1957	1956	1957	1956	1957
8-49	3	0	11	1	5	4
9-46	7	0	12	0	12	3
24-26	4	0	11	0	11	1

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Two crops of turnip varieties were grown in successive years in an experimental garden; the seed for each variety came from the same lot. Twelve plants of each variety were selected at approximately the same stage of development in the two crops. Each storage organ provided three slices for inoculation with histidine-requiring mutants; the remainder of the organ was used to determine its histidine content (Garber, 1958). Although the three varieties in the first year were highly susceptible, they were highly resistant in the second year (TABLE 5). Moreover, the free histidine content of two varieties had greatly changed in the second year (TABLE 6).

Since neither the host varieties nor the pathogens were genetically different in the two years, it was reasonable to assume that nongenetic, that is, environmental, factors may have been responsible for the change from susceptibility to resistance. Since the histidine content of the hosts differed from year to year, it was not unlikely that the concentration of other amino acids could also vary. Consequently, the resistance of the second crop may have resulted from the occurrence of ratios inhibiting the uptake of histidine by the pathogens. This explanation also eliminates the inadequate concentration of this amino acid as a major factor determining the resistance or susceptibility to pathogens requiring histidine.

Another bacterial plant pathogen, *Pseudomonas tabaci*, provided evidence for a third mechanism to explain the avirulence of nutritionally deficient pathogens. This pathogen produces an exotoxin, detectable as a bacteria-free, chlorotic halo surrounding the necrotic focus of infection at the site of inoculation in the leaf of susceptible species and varieties of tobacco (Braun, 1955). Two tryptophan-requiring mutants differed in their virulence when inoculated into the same leaf of a susceptible host (Garber, 1958). The response of the virulent and avirulent mutants to graded concentrations of tryptophan was compared. The virulent mutant exhibited excellent growth over a range of concentrations in which the avirulent mutant showed either poor or no growth. However, both mutants grew equally well in graded concentrations of aqueous leaf extracts, nutrient broth, or yeast extract. Also, both mutants responded equally well when leaf disks were added as auxanographic supplements to pour plates of minimal medium containing the mutants.

TABLE 6

THE MEAN CONCENTRATION AND STANDARD ERROR OF HISTIDINE IN μG./GM. FRESH WEIGHT OF STORAGE ORGAN OF THREE TURNIP VARIETIES IN 1956 AND 1957*

Year	Foliage	White globe	Extra-early purple-top Milan
1956	12.7 ± 1.1	57.1 ± 5.2	40.4 ± 5.3
1957	13.8 ± 1.1	17.9 ± 3.5	71.6 ± 9.1

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Although neither mutant grew in a minimal medium containing acid-hydrolyzed casein known to be deficient in tryptophan, both mutants grew equally well when this medium was supplemented with low concentrations (3 to 5×10^{-5} M) of tryptophan. By adding pools of amino acids to the minimal medium supplemented with low concentrations of tryptophan, it was established that no individual amino acid was effective. However, growth was observed when certain combinations of amino acids were added to the tryptophan-supplemented minimal medium. No growth was detected when pools of vitamins, growth factors, purines, or pyrimidines were used.

These observations suggest that the avirulent mutant could utilize tryptophan as effectively as the virulent mutant but only in the presence of other but nonrequired amino acids. It is reasonable to assume that the amino acids facilitated the uptake of tryptophan by the avirulent mutant. Although supporting evidence is not yet available, it is possible that a membrane transport mechanism may be involved.

Lichstein (1959) has recently discussed the physiological aspects of growth initiation by bacteria. Although *in vitro* conditions were considered, the following quotations are equally relevant to growth initiation *in vivo*. "When a bacterial cell is seeded into a new medium, the factors that will determine whether or not growth will initiate, as well as the rate and extent of growth, are (*a*) the suitability of the physical and chemical environment, (*b*) the availability of the medium components to the cell, namely, permeability, and (*c*) the adaptability of the cell, namely, the ability of its biochemical machinery to handle the new situation via synthesis of essential nutrients, vitamins, coenzymes, and enzymes .... Perhaps the most important environmental agent is the chemical composition of the medium .... One of the more interesting and vexing problems associated with nutrition is that the requirement for a given metabolite may not remain constant, but rather may vary considerably depending on the kind and amount of other components in the medium."

Although the nutritional aspects of the host-parasite relationship have been emphasized, the significance of effective inhibitory mechanisms has not been overlooked or minimized. Effective inhibitory mechanisms probably play a major role in the resistance of hosts to pathogens with relatively few requirements beyond the need for certain elements and an energy source. However, it is also possible that an inadequate nutritional environment may play a significant role in resistance to obligate parasites that must get all their complex nutritional requirements from the host either at the site of inoculation or of localization. It is feasible that the specificity of certain obligate parasites for specific tissues or organs may reflect a relationship between the nutritional demands of the parasite and the availability of the required compounds in these tissues or organs.

Summary

Pathogenicity represents the successful completion of a number of steps in a series of events starting with the entry of the parasite and ending with the manifestation of a disease by the host. According to this scheme, a block at any step may lead to the loss or absence of virulence for the parasite or to resistance for the host, depending on the source of the block. The nutrition-inhibition hypothesis of pathogenicity specifically considers the multiplication and metabolism of the parasite after entry. The nutritional requirements of the parasite must be satisfied at the site of entry or of localization. Experimental evidence indicating that the host may or may not be providing specific nutrilites for the parasite has come from studies using nutritionally deficient mutants of animal and plant pathogens, including bacteria and fungi. Three different mechanisms were found to be responsible for the avirulence of nutritionally deficient pathogens, and examples of each have been presented and briefly discussed.

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BIOCHEMICAL ASPECTS OF VIRULENCE OF *HEMOPHILUS INFLUENZAE**

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The pioneer studies of Pittman[1] on variation and type specificity in *Hemophilus influenzae* established that these bacteria, which require diphosphopyridine nucleotide and heme for growth, may be divided into two broad groups: one, encapsulated and exhibiting an iridescent growth on transparent agar; the other, nonencapsulated or nontypable, showing no iridescence. Strains producing iridescence are divisible into six serological types: *a*, *b*, *c*, *d*, *e*, and *f*; the noniridescent, nontypable strains, on the other hand, are serologically a heterogeneous group.

The pathogenic potentiality or virulence of *H. influenzae* for man and mouse is related, in part at least, to the presence of a capsule and, for man, to the age of the host. Various authors[1-4] have reported that an encapsulated or typable strain is more virulent for mice than a nonencapsulated strain. In man, the pathogenic role of encapsulated *H. influenzae* is clearly established. In the pediatric age group, type *b* is one of the most common causes of serious infections; the clinical patterns seen most frequently are meningitis, pyarthrosis, obstructive laryngitis, pneumonia, and otitis media. Bacteremia is a characteristic feature of each variety. The same type is known to cause all of these infections in adults, but the incidence is much lower. This decline of virulence with the age of the host is related to the production of antibodies after infection with these organisms. Fothergill and Wright[5] have shown that the age incidence of meningitis due to type *b* is inversely correlated with the age incidence of the bactericidal power of the blood for type *b*. Tunevall[6] has reported a corresponding age incidence relative to complement-fixing antibodies against nonencapsulated strains as well.

Why type *b* causes more than 95 per cent of acute *H. influenzae* infections in infants and children is unknown. Alexander *et al.*[7] have shown that the protective or curative power of rabbit antiserum to type *b* resides in the anticapsular antibody; in their experiments essentially all of the protective power of the serum for mice was removed when virtually complete absorption of this antibody was effected. A part at least of the unique virulence of type *b* may, therefore, lie in the chemical nature of its capsular antigen.

Hence a chemical study of the specific soluble substances of the different specific types of *H. influenzae*—with the exception of type *e*—was undertaken. The purification and analysis of the capsular substances of types *a*, *b*, *c*, *d*, and *f* was carried out by MacPherson *et al.*[8] and the authors concluded that they were all polysaccharides. It was reported that all of the soluble specific substances except that from type *d* contained organically bound phosphorus. A type *b* preparation showed a positive orcinol reaction.

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More recently, Zamenhof *et al.*[9] have reported the capsular substance of type *b* to be a polymer of ribophosphoric acid or polyribophosphate rather than a polysaccharide. Crystalline ribonuclease was found to effect a slow breakdown of this polymer. Zamenhof postulates that the structure is a polyribophosphate chain connected by 3,5 phosphoric acid ester bridges; to this chain a similar chain is linked in 1,1′ glycosidic linkages. According to Zamenhof, the postulated structure thus bears a resemblance to the postulated structure of ribonucleic acid. Zamenhof *et al.* also re-examined the capsular substance of types *a*, *c*,[10] and *d*, and Rosenberg and Zamenhof[11] that of type *f H. influenzae.* TABLE 1 summarizes results relative to the type of sugar present, the ratio of sugar to phosphate, and the probable chemical nature of the capsular specific substance. The nature of the sugar component was determined by paper chromatography. Type *d* is the only one reported that lacks phosphate. The type *f* substance is an acetylated galactosamine to which phosphorus is bound in the ratio of 2 sugar molecules to 1 phosphorus atom; the other polysugar-phosphates show a 1:1 ratio. Williamson and Zinneman[12,13] reported that there are two type *e* capsular antigens: *e*1, which is superficial, and *e*2, which is deeper. Strains of type *e* exhibited partial degradation from the *e*1-*e*2 type to *e*2 type alone, but this partial degradation did not lead to any demonstrable change in infectivity for white mice.

TABLE 1

SPECIFIC CAPSULAR SUBSTANCES OF FIVE TYPES OF *H. INFLUENZAE*

Type	Sugar component*	Specific capsular substance	Sugar / Phosphorus ratio
a	D-glucose	polyglucophosphate	1:1
b	D-ribose	polyribophosphate	1:1
c	D-galactose?	polygalactophosphate	1:1
d	hexose	? polysaccharide	No P
f	galactosamine	polygalactosamine phosphate	2:1

* Sugar determined by paper chromatography.

With this knowledge, we are able to define in more exact chemical terms the end product of DNA-mediated transformation to type specificity in *H. influenzae.* Tunevall,[14] using the agar gel precipitation method, has reported that encapsulated *H. influenzae* cells created from nonencapsulated strains in transformation experiments revealed no antigenic differences from their parent strains except that they possessed a component identical with the specific capsular substance. From this it may be inferred that DNA-mediated conference of type specificity generally does not entail other antigenic changes in the transformed cell. A possible exception is our demonstration that nonencapsulated cells derived from type *b* cells (R*b*), when exposed to the DNA from natural type *a* cells, are changed extremely rarely to cells reactive with both types *a* and *b* antisera.[15] These cells, labeled S*ab*, produce less of the type *b* capsular substance than of type *a* substance. Quantitative studies showed a 27-fold excess of hexose relative to ribose in a single preparation of the capsular substance of S*ab* cells. These cells appear to have the genetic potentiality of producing normal amounts of polyribophosphate, since those variants that have lost the capacity to synthesize detectable amounts of polyglucophosphate are able to produce normal amounts of polyribophosphate, as measured by capsular swelling and the precipitin reaction. Also, when appropriate recipient populations are exposed to the DNA from S*ab* cells, three types of encapsulated cells are formed: types *a*, *b*, and *ab*.[15]

In our laboratory, antiserum to the *ab* type was produced. The antibody that reacted with the *b* component of S*ab* cells was easily removed with absorption with type *b* cells, leaving the type *a* antibody readily demonstrable. By removal of the type *a* antibody with repeated absorptions with type *a* cells, type *b* antibody could still be demonstrated. Absorption of the serum with *ab* cells removed both types of antibody. Two separate and distinct types of capsular substance, but not a single substance reactive with both types of antibody, therefore appear to be synthesized by these cells. Their virulence for mice will be discussed subsequently.

Bacterial cells that are changed to a new type or from a nonencapsulated to a type-specific form by transformation are generally monotypic. It was of interest, then (1) to determine whether the cell transformed to a single type is more virulent than the nonencapsulated recipient cell before transformation; (2) to determine whether the source of the recipient cell is a factor in virulence; and (3) to ascertain whether cells transformed to type *b* are more virulent than those transformed to other types. To these ends, the virulence in mice of nonencapsulated populations used as recipient populations was compared with that of the natural type from which they were derived and with that of the type to which they were transformed. To insure a greater uniformity of iridescence and capsular swelling in the type-specific populations, cells from freshly selected single colonies were used in each experiment.

The yield from a young (4½ hour) Levinthal plate culture was suspended in nutrient broth and diluted to contain approximately 2×10^9 viable cells per milliliter. This density gave a reading of 100 to 107 with the Klett-Summerson colorimeter (filter No. 54). Appropriate dilutions of organisms were suspended in 7 per cent aqueous mucin adjusted to *p*H 7.2 with NaOH and containing 1 per cent glucose. A single lot of mucin* was used. Swiss albino mice weighing 13 to 15 gm. were inoculated intraperitoneally with 1 ml. of the mucin suspension of organisms. Most deaths occurred within 24 to 48 hours of inoculation.

A single strain of natural types *a*, *b*, and *d*, their nonencapsulated variants R*a*, R*b*, R*d*, and certain of the latter transformed to types *b*, *ab*, and *d* were selected for study. Types *a*, *b*, and *d* were selected for two reasons: (1) their capsular substances are a polypentosephosphate, polyhexosephosphate, and a phosphorus-free hexose, respectively; and (2), strains of types *a* and *d* in one report[16] were stated to be virtually avirulent for the mouse whereas type *b* strains are known to be more virulent than their nonencapsulated variants.[1,3] Type *b* was also selected because of its predominance in acute infections in infants and children.

The data in TABLE 2 show that under the experimental conditions used the natural types *a* and *d* strains selected were more virulent than the type *b* strain.

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* Wilson Laboratories, Chicago, Ill.

In subsequent experiments one or two cells of the type *d* strain were lethal. Uniformly, the dose of type *b* organisms required to kill at least 50 per cent of the mice was 100-fold greater than this. However, the strain of type *b* used in experiments reported upon by us earlier[7] was comparable in virulence to the types *a* and *d* strains used in the present experiments.

These data confirm that a natural encapsulated strain of *H. influenzae* is more virulent than its nonencapsulated variant, but the latter is not avirulent for the mouse. As reported in the literature,[3] there is a greater irregularity in virulence with the nonencapsulated forms, an irregularity of such degree that in these and other experiments the LD_{50} was not calculated. It is apparent, however, that the encapsulated strain is at least tenfold more virulent than the nonencapsulated.

TABLE 2

VIRULENCE FOR MICE OF STRAINS OF THREE NATURAL TYPES OF *H. INFLUENZAE* AND THEIR NONENCAPSULATED VARIANTS

Strain	Dead/total ratio for inoculum size					
	200,000	20,000	2000	200	20	2
S*a*				6/6	5/6	3/6
R*a*		3/6	5/6	1/6	1/6	0/6
S*b*			5/6	5/6	1/6	
R*b*	5/6	3/6	4/6	2/6		
S*d*			6/6	6/6	6/6	
R*d*	6/6	4/6	4/6	3/6		

S, encapsulated. Small letter refers to specific type. R, Nonencapsulated. Small letter refers to type of origin. S*b*, R*b*, S*d*, R*d* compared simultaneously.

TABLE 3

VIRULENCE OF NONENCAPSULATED VARIANTS OF THREE TYPES OF *H. INFLUENZAE*

Strain	Experiment	Dead/total ratio for inoculum size					
		200,000	20,000	2000	200	20	2
R*a*	1		3/6	5/6	1/6	1/6	0/6
	2		6/6	3/6	3/6	3/6	1/6
R*b*	1	5/6	3/6	4/6	2/6		
	2		2/6	3/6	0/6	2/6	1/6
	3		6/6	3/6	2/6	2/6	1/6
R*d*	1	6/6	5/6	6/6	5/6	4/6	
	2		6/6	6/6	5/6	1/6	3/6
	3	6/6	4/6	4/6	3/6		

Experiment 2: R*a*, R*b*, and R*d* tested simultaneously.

Although the data in TABLE 2 indicate that there is no significant difference in virulence among the nonencapsulated types, results from all experiments in which they were used (TABLE 3) suggest that strain R*d* is, in general, more virulent than strain R*b* and that strains R*b* and R*a* may be comparable in virulence. The irregularity of virulence of all strains is manifest, but approximately 200 organisms of strain R*d* were lethal for at least 50 per cent of the mice in all three experiments; a tenfold higher dose of R*b* cells was required for this endpoint. Since cultures of the heart's blood of those dead mice examined showed the type of growth characteristic of the nonencapsulated forms, reversion to specific type does not appear to function in the virulence of these strains.

The apparent greater virulence of the R*d* population may explain the greater virulence of types *b* and *d* conferred on R*d* populations when compared with that of the same two types conferred on R*b* populations by DNA. The data in TABLE 4 are compiled from experiments that compared the virulence of R*b* and R*d* cells transformed to types *b* and *d*. The nonencapsulated recipient population and natural encapsulated population used as the source of DNA are included for comparison. Type *b* created in R*b* populations is approximately tenfold more virulent than the natural type *b*. Titration of the supernatant of a 48-hour culture of these two populations with type *b* antiserum did not reveal a gross difference in the amount of capsular material released from the cells. The virulence of type *b* may, therefore, have been enhanced by the transformation process. In a total of three experiments, types *b* and *d* conferred on R*b* populations were approximately tenfold more virulent than the natural type *b* strain.

TABLE 4

VIRULENCE OF R*b* AND R*d* CELLS TRANSFORMED TO TWO TYPES OF *H. INFLUENZAE*

Strain	Dead/total ratio for inoculum size				
	20,000	2000	200	20	2
R*b*	6/6	3/6	2/6	2/6	1/6
S*b*			5/6	1/6	0/6
$\mathrm{S}b_{(\mathrm{R}b)}^{*}$			6/6	4/6	1/6
$\mathrm{S}d_{(\mathrm{R}b)}^{*}$			6/6	6/6	1/6
R*d*	5/6	6/6	5/6	4/6	
S*d*			6/6	6/6	6/6
$\mathrm{S}b_{(\mathrm{R}d)}^{*}$			6/6	6/6	6/6
$\mathrm{S}d_{(\mathrm{R}d)}^{*}$			6/6	6/6	6/6

* Strains of transformation origin. Recipient population in parentheses.

The natural type *d* strain and the R*d* strain transformed to types *b* and *d* were so highly virulent for mice that differences could not be detected. It is apparent, however, that types *b* and *d* conferred on R*d* populations are approximately tenfold more virulent than the same types created in R*b* populations. This finding was confirmed in an experiment in which the virulence of types *b* and *d* formed in both R*b* and R*d* populations was examined simultaneously. Thus transformation to homologous or heterologous specific type results in an increase in virulence, but the recipient cell itself appears to function in the virulence. We have been unable to show by the semiquantitative procedures mentioned earlier that this function is related to the amount of capsular material produced.

The virulence of the di-type or S*ab* cells was of interest since they could be created in type *b* populations as well as R*b* and R*d* populations. To recapitulate, these are cells endowed with the capacity to produce both polyribophosphate and polyglucophosphate. The data in TABLE 5 show that the *ab* type created in R*d* populations [S*ab* (R*d*)] is at least tenfold more virulent than the same di-type created in R*b* populations [S*ab* (R*b*)] and 10,000-fold more virulent than that created in S*b* populations [S*ab* (S*b*)]. The low virulence of S*ab* (S*b*) was confirmed in another experiment; in one of three experiments, the virulence of S*ab* (R*b*) was found to be as low as that of S*ab* (S*b*). Thus the transformed cell may also be less virulent than its parent population.

We were unable to detect any significant differences in the amount of type *a* substance elaborated by the three different S*ab* populations and by the natural type *a* population. The amount of type *b* capsular substance formed by S*ab* cells is less than that formed by natural type *b* populations.

TABLE 5

VIRULENCE OF S*ab* CELLS PRODUCED IN THREE STRAINS OF *H. INFLUENZAE* POPULATIONS

Strain	Dead/total ratio for inoculum size				
	20,000	2000	200	20	2
$\mathrm{S}ab_{(\mathrm{R}b)}$	5/6	4/6	4/6	2/6	
$\mathrm{S}ab_{(\mathrm{S}b)}$	3/6	2/6	1/6	1/6	
$\mathrm{S}ab_{(\mathrm{R}d)}$		6/6	6/6	5/6	3/6

Cultures of the heart's blood of mice that died from infection with the di-type created in S*b* or R*b* cells commonly yielded only a few colonies or no growth. On the other hand, a heavy growth of S*ab* cells was generally cultured from those mice that died of infection with the di-type created in R*d* populations. This difference in degree of bacteremia was noted in the natural types *b* and *d* populations but to a much lesser extent.

Summary

Of the five specific types of *H. influenzae* studied, type *b* is the only one that contains a pentose (ribose) as the sugar component of its capsular substance; either a hexose or hexosamine is the sugar component of the four other types examined. All but type *d* are polysugarphosphates. Type *b* is unique, therefore, not only in its pathogenic potentiality for infants but also in the chemical nature of its capsular substance, a polyribophosphate. Mucin-enhanced virulence tests in mice, however, have not revealed its uniqueness; type *b* of natural or transformation origin was not more virulent than the other types examined.

Nonencapsulated cells transformed to specific types by DNA are in general more virulent than the cells before transformation. Differences in degree of virulence noted appear to be related to the source of the recipient population.

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Discussion of the Paper

Robert Austrian (*Downstate Medical Center, State University of New York, Brooklyn, N. Y.*): Some comments about pneumococcus may be pertinent. It is well recognized that different capsular types of pneumococcus manifest different degrees of virulence in different mammalian species and that these differences in virulence may be related in part to the chemical composition of the several capsular polysaccharides. In man the preponderance of pneumococcal infections is caused by organisms of the first eight numerical types, but even these types show dissimilar degrees of invasiveness. In recent months, strains of penumococcus type I and type VI respectively have been isolated from an equal number of hospitalized patients, but there has been a striking difference in the bacteremia rates in persons yielding one or the other of these organisms. Pneumococci were recovered from the blood of 33 per cent of the patients harboring pneumococcus type I but from the blood of none of those patients from whom pneumococcus type VI was isolated. Of possible relevance to Leidy's comments is the fact that ribitol-PO_4 is a component of the capsule of pneumococcus type VI. This organism appears also to be tolerated well by mice, in which it causes chronic bacteremia as pointed out by Erna Mørch.[1]

It has been surmised in the past that some relationship between the composition of capsular carbohydrates and tissue polysaccharides may influence the interplay between host and parasite. For example, it is known that pneumococcus type XIV, a frequent cause of infection in infancy, has a capsule that is related chemically to human blood group substances. Recently E. E. B. Smith and G. T. Mills in our laboratory found fucose, a component of human blood group antigens, to be a constituent of type I pneumococcal capsular polysaccharide. In animal species other than man, increased susceptibility to infection with specific pneumococcal types has also been observed. Epizootics in guinea pigs are caused most frequently by pneumococcus type XIX, and spontaneous outbreaks of infections with pneumococcus type II have been observed in several colonies of rats. The question of the biochemical relationship of capsular polysaccharides to tissue components is a subject which merits further scrutiny.

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FACTORS OF VIRULENCE OF *VIBRIO CHOLERAE*[*]

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It has been 76 years since Robert Koch's announcement of the isolation of *Vibrio cholerae* (*V. comma*). In this span of time, rather satisfactory progress has been made in detection and identification of the pathogenic vibrios, in defining a physiological basis for treatment and in prevention of the pandemics of the last century. In their justifiable preoccupation with the foregoing problems, microbiologists have been less successful in describing the essential lesion of Asiatic cholera and its pathogenesis or in identifying the biological and biochemical attributes peculiar to the pathogenic vibrios. Nevertheless, a certain concept of cholera pathogenesis has achieved the status of a textbook dictum: *V. cholerae*, confined within the intestinal lumen, is believed to act through the agency of its enzymes and toxins to denude the villi of their protective epithelial covering and to effect a profound alteration of permeability of the underlying capillaries; there ensues an outpouring of exudate, with consequent dehydration, electrolyte imbalance, and shock, sometimes accompanied by ischemic or toxic renal damage.[3]

The discovery of mucinolytic enzymes in *V. cholerae* filtrate by Burnet and Stone[4,5] appeared to supply the agency required to produce the epithelial desquamation in the small bowel that so often has been observed at autopsy.[1] These enzymes were found to depolymerize glandular mucosubstances and to cause shedding of epithelium in the resected gut of man and experimental animals.[4-7] Interest in the mucinases in relation to cholera has focused upon the "mucinase I" of Burnet and Stone rather than upon "receptor destroying enzyme" ("neuraminidase"), since the former produces more extensive depolymerization and hence might liquefy the protective mucous covering of the epithelium and detach the latter from its basal membrane.[4,5,8] It is worth recalling, perhaps, that Burnet and Stone ascribed the epithelium-shedding activity in part to a third filtrate factor, the "tissue disintegrating enzyme." These investigators also emphasized the role of tissue autolytic processes in initiating the desquamating action of both factors on the gut segments.[4]

Confirmation[6] of this discovery came promptly, stimulated perhaps by a certain teleologic appeal, and evidence accumulated for the projected role of the mucinases in pathogenesis of cholera. With either the guinea pig ileum strip[6,7] or ovomucin[8] as substrate, it was found that mucinase is produced by all smooth *V. cholerae* cultures,[7,9-13] that its activity is neutralized by antiserum to culture filtrate,[4,5,8,10-12] that one or both of at least two serologically different mucinases may be produced by one strain,[11,14] that mucinase is present in the bowel contents in fatal guinea pig enteric infections,[15] and that epithelial detachment is found in some animals dead of experimental cholera.[16-18] Freter found that active[15] but not passive oral immunization against culture

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[*] In general, only more recently emphasized aspects of the problem will be considered in this paper. The reader is referred to the exhaustive monograph of Pollitzer[1,2] for material not covered in this report.

filtrate protected guinea pigs against fatal infection. Lam and his colleagues[21] reported that mucinase-containing culture filtrates, injected into isolated segments of normal mouse intestines, either excised or *in situ*, caused an influx of fluid and increased intralumen pressure, an effect reminiscent of that produced by Burrow's phospholipid toxin.[20] Active immunization with filtrate or passive immunization with its antiserum prevented this phenomenon. A striking toxicity of *V. cholerae* filtrates, usually associated with mucinase and neutralizable by filtrate antiserum, was described by Chugh *et al.*[14] This thermolabile toxic activity was manifest by dermonecrosis in rabbits and guinea pigs and by lethal effect in mice, guinea pigs, rabbits, monkeys, and the embryonated egg. Unfortunately no purified mucinase has been available to test its association with these activities of *V. cholerae* filtrates. High titer mucinase, relatively free of RDE, has been produced in aerated cultures in a defined medium[13] in our laboratories; thus its purification may be feasible.

Mucinase is produced to low titer by some strains of *Shigella* and *Salmonella*[22,23] and by *Escherichia coli*, more frequently by enteropathogenic strains of the latter.[23] An association of high titer mucinase production with virulent *V. cholerae* is suggested in reports that noncholeragenic vibrios produce low titers only,[24] and that R *V. cholerae* produces little or none.[7,25] However, the latter correlation could not be confirmed in our laboratories with S and R strains isolated from the same patients,[13] and others have reported high titer mucinase from El Tor and nonpathogenic water vibrios.[9,11,14] Hence mucinase production is not limited to pathogenic vibrios. Moreover, there is yet no documented evidence for its production in human infections. Doubt as to its postulated role as an epithelium-desquamating factor in human infections during life has been raised by the recent report of Gangarosa and his colleagues,[26] who found atrophic, although intact epithelium, sometimes with adherent mucus, in all biopsy specimens collected with the Crosby capsule from the stomach, duodenum, jejunum, upper ileum, and colon of cholera patients. These and other investigators consider epithelial detachment to be a postmortem artifact. Thus any contribution of mucinase to pathogenesis of cholera remains to be demonstrated, and its possible role may be related to activities other than epithelial desquamation.

V. cholerae is known to secrete an imposing array of other digestive and depolymerizing enzymes,[9] although none has been associated with pathologic effects and most have their counterparts in pancreatic secretions and the succus entericus.

Since cholera infection appears to involve primarily the small intestine in man, it is of paramount importance to determine how it becomes established and proliferates there. Efforts to infect experimental animals have been based upon the assumption that the vibriocidal gastric acidity must be neutralized, that the transit time of an inoculum in the small intestine must be extended, and that a presumed antagonistic bacterial flora must be eliminated. To these ends, animals have been dosed with $CaCO_3$ and opium, starved, and treated with antibiotics. The postulated protection by a normal bacterial flora is supported by Freter's[27] observation that a simultaneous dose of *E. coli* protects guinea pigs from an otherwise fatal infection with *V. cholerae* and that it suppresses nonfatal, persistent infection by *V. cholerae* or *Shigella* in guinea pigs and mice.[28,29] If comparable microbial antagonism were involved in untreated experimental animals or in man, then it might be assumed that resistance would be in part a function of the effectiveness of such flora and, conversely, that invasiveness of *V. cholerae* strains might be related to their capacity to compete with and displace the normal flora. Above average tolerance for gastric acidity or greater resistance to the vibriocidal activity of saliva[30] also might confer greater invasive capacity to a strain of cholera vibrio. These possibilities appear worthy of more thorough investigation.

Actually, little evidence supports the concept of antagonism to *V. cholerae* by a normal flora of the small intestines. Numerous recent studies, on the contrary, indicate that the lumen of the small bowel of man is remarkably free of bacteria, and especially from *E. coli*[31,32] except possibly in acholorhydria, which is believed to be conducive to cholera infection.[33,34] In mice, guinea pigs, and rabbits, also, *E. coli* is absent in the small intestines or present only in small numbers, except in the cecum.[35-37] The 4- to 5-day starvation period necessary to lethal *V. cholerae* infections in the guinea pig[15] was reported to promote extension of *E. coli* into the small bowel of the rat.[35] Even the protection afforded by *E. coli* fed to animals infected with *V. cholerae* and *Shigella* may be mediated in part by factors other than active antagonism or competition. Schaedler and Dubos,[38] for example, have found that the establishment of enteric residence in mice by a specific *E. coli* strain enhances their resistance to parenteral infection with nonenteric pathogens. An "antagonism" by *E. coli* to *V. cholerae* infection in the embryonated egg was demonstrated by Finkelstein and Ransom[39] to result from a prior modification of the host's resistance by endotoxin. Starvation, too, may decrease resistance to infection[40-42] and to endotoxin[43] through impairment of the host's general defense mechanisms or injury to the intestinal mucosa. The picture of acute enteritis in the biopsy specimens of Gangarosa *et al.*[26] was found to be superimposed upon a chronic enteritis suggestive of malnutrition, in support of earlier claims that malnourished individuals, especially those with concurrent infections, are prone to cholera.[1,33]

A recent report of the rapid mechanical expulsion of bacteria injected into the small intestines of rats, in the absence of significant bactericidal effects,[44] raises the question of how any microorganism can become established and proliferate in this relatively bacterium-free environment. It may be speculated that either the downward propulsion mechanism may fail or that certain bacteria may possess a mechanism for firm attachment onto or within the epithelium, there to flourish and shed their excess numbers into the lumen. Taylor and his colleagues[37] observed that the more virulent strains of *E. coli* inoculated into ligated intestinal loops of rabbits (technique of De and Chatterjee, 1953)[45] adhered in a compact layer over the epithelium covering the villi, whereas the less virulent strains grew free in the lumen. Schaedler and Dubos[38] found that the establishment of residence of their resistance-enhancing strain of *E. coli* in mice was associated with resistance to removal by washing the excised gut. A possible adherence mechanism is offered by the fimbriae, or pili, found under appropriate cultural conditions on many enteric bacteria.[46-48] Although there is no electron microscopic evidence of these structures on *V. cholerae*, several strains grown under suitable conditions have been found to agglutinate strongly the red blood cells of man, guinea pigs, and rabbits, to adhere to guinea pig intestinal epithelial cells, and to agglutinate atypically in homologous O antiserum. To carry this speculation further, it may be imagined that mucinase might promote spreading of the growing organisms over the epithelial surface. Epithelial attachment of *V. cholerae* also might account for its persistence during the acute phase of the disease when coliforms and other colonic flora appear to have been flushed out by the copious flow. Finally, disposition of *V. cholerae* in or on the epithelium might explain its phenomenally rapid clearance during convalescence. Possibly, as Burrows and Havens[49] suggested, antibody is transported to the mucosa by lymphocytes and plasma cells, there to sensitize the vibrios to phagocytosis by macrophages, found in abundance in the human infection by Gangarosa *et al.*[26] and in the rabbit intestinal loop by Jenkin and Rowley.[50]

There is yet no complete agreement on the nature of *V. cholerae* toxin or on its site and mode of action. Perhaps, as Burrows[51] has suggested, the several toxic materials obtained in different laboratories all may be moieties, some fragmentary, of the classical endotoxin complex. Gallut,[52] Freter,[53] and others obtained a major portion of the total endotoxicity in a lipopolysaccharide component, while Jenkin and Rowley[54] found in their strains only small and widely varying quantities of this fraction. The toxicity that they recovered resided primarily in the glycoprotein of the Boivin complex, while species and type specificity of the antigen were determined by the polysaccharide component.[55] This and prior studies indicate that the endotoxin complex may be identified with the O antigen. Takaishi[56] recently presented evidence that the O antigen of the cholera and the water vibrios is a cell wall component, although toxicity and immunogenicity were associated with a cytoplasmic constituent as well.

Opposition has appeared to the hypothesis that cholera toxin alters capillary permeability in the human infection, leading thereby to massive exudation. Several investigations have failed to demonstrate a significant protein component in cholera stools or a transfer of Evans blue-albumin complex through the capillary endothelium and the mucosa.[57-59] On the basis of their studies of cholera physiopathology in Bangkok and of those of Visscher *et al.*[60] on intestinal excretion and absorption of water and electrolytes, Watten and colleagues proposed, as had Saha and Das,[58] that the fluid loss in the stool may be a transudative process or even an increased normal excretion coupled with impaired water and electrolyte reabsorption in the colon. Whatever the nature of the lesion resulting in diarrhea, there is no better agreement as to the agency or mechanism that produces it. Whereas Freter[53] was unable to produce pathologic effects in guinea pigs with massive oral doses of heat-killed cells or with his acid soluble lipopolysaccharide toxin, Dutta *et al.*[17] obtained in suckling rabbits a fatal choleralike diarrhea with Gallut's "four-hour" glycolipid toxin.[52] De[61] obtained fluid distention and an exudate "characteristic" of cholera in the ligated intestinal loop of rabbits with 1 ml. of an 18-hour peptone water culture filtrate. Jenkin and Rowley,[62] however, could not induce distention with their toxin preparation or with streptomycin-sterilized contents from a positive-infected loop, and Ghosh and Mukherji[63] failed to obtain loop distention with ultrasonic extracts of *V. cholerae*. The induction of distention in the intestinal loop by lactic acid-producing microorganisms and by lactic acid alone (Jenkin and Rowley)[62] introduces a new concept of pathogenesis in this static *in vivo* culture system. Whether lactic acid may play a role in human cholera or in fatal enteric *V. cholerae* infection in the guinea pig or the suckling rabbit is conjectural at this time.

The question of a virulence differential of Ogawa and Inaba serotypes continues to arise, although Pollitzer[1] states that it is now generally accepted that no consistent difference in mortality occurs in epidemics due to these two serotypes. The virulence of a few strains of each serotype has been compared by intraperitoneal mouse inoculation of mucin suspensions[64,65] and of toxin preparations, but none of these studies has been based upon an adequate number of strains to permit any conclusions, despite claims for greater virulence[65] or toxicity[66,67] of one or the other serotype. Nor does evidence exist for any consistent difference in mouse virulence between strains of *V. cholerae* and of El Tor and other water vibrios.[68] Jenkin and Rowley[54] found no important difference in mouse toxicity of the glycoprotein derived from five *V. cholerae* and three water vibrio strains. Thus no specific virulence factor has been identified that can account for the wide difference in human pathogenicity of the choleragenic and the noncholeragenic vibrios.

Differences in virulence between antigenically smooth (S) and rough (R) strains of *V. cholerae* appear to be far more consistent and clear-cut than between any antigenically smooth varieties of vibrios. Loss of the group- and type-specific O antigens, whether by variation *in vitro* or in the human host, is associated with a striking decrease in mouse virulence[64,69] and almost complete loss of virulence for the 13-day chick embryo.[70,71] Although R strains may appear in the stools of convalescent patients,[69] there is no evidence that they are capable of producing disease. In human, as in the experimental infections mentioned, the O antigen appears to be essential to virulence. This role is mirrored to a degree in protection afforded by active immunization with heat-killed S cells or by passive immunization with corresponding antiserum. Jenkin and Rowley,[50] for example, report that distention of the ligated rabbit intestinal loop was prevented by immunization with heat-killed *V. cholerae* but not with a water vibrio that shared the same toxic protein component of the Boivin complex but differed in the specificity of the polysaccharide moiety. Takaishi[56] found immunogenicity to be associated with cell wall fractions of *V. cholerae* containing the O antigen but he, like Burrows,[68] found protective antibody to be elicited by other cellular components as well. Differences in the qualitative and quantitative chemical composition of S and R strains of *V. cholerae* are indicated in recent studies by Burrows[72] and by Singh and Devi,[73] but how these chemical differences may be related to antigenicity, immunogenicity, or virulence is yet to be determined.

V. cholerae is notorious for its tendency to rapid variation in stock cultures, and it is not unlikely that many of the divergent reports in the literature have their basis in this fact. In an effort to obtain unaltered strains for comparative studies, Burrows and I collected a series of cultures in Calcutta in 1953 and preserved them, within a few culture cycles after isolation, by lyophilization and in the frozen state at −70° C.[69] Even with these precautions, some variation in these cultures is not improbable, although their colonial and antigenic homogeneity has been well maintained by comparison with other collections studied. Some of these strains have been used to compare mouse virulence in Burrow's laboratory,[69] virulence for the 13-day embryonated egg in my laboratory,[70,71] and toxicity for mice by Gallut.[74] Representative data (TABLE 1) suggest that in each test system a different determinant of virulence of the S strains may be dominant. The R strains, however, are clearly of lesser virulence and lower toxicity in each test system. Daily S isolates from a single case sometimes showed fluctuations in LD$_{50}$ values, the relations of which were reversed in the different test systems. While Gallut[74] saw evidence in his data of a trend to maximum toxicity of isolates from the third day of disease, Husain and Burrows[69] observed in certain cases a progressive trend to increased virulence of fourth- or fifth-day S isolates. Gardner,[70] on the contrary, found the first isolate from each case generally to be the most virulent for the chick embryo. The few available strains from fatal cases possessed a greater potential for invasion and proliferation in the circulatory system of mice and produced a lower mean LD$_{50}$ for the embryonated egg than that of strains from nonfatal cases. Burrows[72] reported also that strains from fatal cases were characterized by greater amounts of an intracellular polysaccharide component and of a cell wall protein in the more mobile of his electrophoretic cell fractions. While it is obvious that no significance can be attached to these

TABLE 1
COMPARISON OF MOUSE- AND CHICK-EMBRYO VIRULENCE AND MOUSE TOXICITY OF CALCUTTA STRAINS
Sample Data

Case and culture No.*	LD$_{50}$		Mouse LD$_{50}$ doses/ml. toxin[74]
	13-day chick embryo[70]	Mouse[69]	
C-21 C-22 C-23 C-24	1,200,000 1,700,000 1,000,000 1,000,000	124,000 98,666 43,918 11,917	18.1 10.0 50.0 11.2
C-141 C-142 C-143 C-144 (died)	46,000 540,000 200,000 36,000	300 420 189 50	17.2 23.8 25.6 18.5
C-371 C-372 (died)	6,000 170,000	45 38	— —
C-414 R†	>1,700,000,000	1,810,000	4.0
C-381 C-383 C-385 R C-386	1,800,000 2,000,000 >1,800,000,000 3,300,000	93 136 1,030,000 95	— — — —

* Last digit of culture number indicates day of hospitalization on which culture was isolated; first digit, or first two digits, indicate case number.

† R, antigenically rough isolate.

suggestive but inadequate data, the methods for comparing virulence of strains and mutants appear promising.

An association of intraperitoneal mouse virulence of *V. cholerae* with nutritional prototrophy, similar to that demonstrated for *Salmonella typhosa* by Bacon *et al.*,[75] was found by Formal and his colleagues[76] with a purine-dependent stock strain and its prototrophic mutant. Bhaskaran and Rowley[77] had reported earlier that 50 per cent of 158 *V. cholerae* strains that they examined were purine-dependent and implied that the higher purine content of human and guinea pig sera might be related to the role of man as a natural host and of the guinea pig as an artificial host. Formal's study, however, failed to show a significant difference in virulence of the prototroph and the auxotroph in fatal enteric guinea pig infections. Finkelstein[78,79] in our laboratory found that all of more than 100 strains from several epidemic sources, preserved by lyophilization, were prototrophic, that purine dependence in stock strains often was associated with obvious colonial and/or antigenic degradation, and that agar passage could lead to selection of auxotrophs. It seems probable that while purine and methionine dependent mutants are selected readily *in vitro*, they possess no selective advantage in the human host.

Most stock cultures of *V. cholerae* that I have examined on glycerol-glucose agar ("2-1 agar") by the Henry oblique light technique[80] were found to be mixtures of colony types or pure types differing one from the other. In contrast, Burrows and I found in Calcutta, India, that nearly all colonies in primary culture from all patients and at all stages of the disease were of a single characteristic colony type designated by Husain and Burrows[69] as "granular chromatic" and in our studies as "typical, opaque striated." The same type was found consistently in the 1958 Bangkok, Thailand, epidemic, with the qualification that a small proportion of less opaque or even translucent colonies sometimes made a transient appearance in the primary cultures. In static or serially transferred cultures of the "typical" colony type, examination by the Henry technique usually reveals rapid selection for progressively less opaque colony types, then for dwarf colonies, rough "prismatic" colonies composed of elongated or filamentous cells and, eventually, for many other colony types.[81] It seems reasonable to assume that the environment of the human intestine strongly selects against these "atypical" colony types, which so often characterize stock culture collections, and that human virulence is somehow associated with the "typical" colony form.

No constant association of qualitative antigenic structure with colony type has been found and, conversely, antigenic variation may occur without visibly associated colonial modification; the two characters vary independently. Antigenically R colonies found in convalescents or in culture may have the same visible colonial characteristics of the parent S form, while colonially rough, even subtilislike variants, may possess the O antigens of the parent S culture. Nevertheless, colonial variation even of the most trivial degree seems associated with loss of survival value in the natural infection and is linked with a change in virulence for the embryonated egg (TABLE 2). For example, variation leading to progressive loss of opacity on 2-1 agar, even without antigenic change detectable in the sensitive "homologous serum-soft agar" system,[82] is accompanied by loss of virulence. The degree of opacity or refractility on 2-1 agar was found to be correlated with the quantity of sudanophilic lipid stored as granules or globules within the cell. Since most of this lipid is ether-soluble and chloroform-insoluble, it appears to be neutral fat. This lipid probably has no direct relation to virulence, since it is not found in vibrios in the cholera stool nor in virulent cells grown on nutrient and infusion agars. Possibly its presence in cells grown on glycerol-glucose agar may be but one overt expression of a virulence-associated metabolic potential that is diverted to other syntheses in the infection.

TABLE 2

LD_{50} OF COLONIAL VARIANTS IN 13-DAY EMBRYO: CALCUTTA STRAINS (1953)

Sample Data

Strain*	Description	LD_{50}
C-371 (original)	Typical, opaque, striated	6,000
C-371-A_7	Slightly less opaque	510,000
C-371-B_3	Semi-translucent	>29,000,000
C-381 (original)	Typical, opaque, striated	1,800,000
C-381-B_3	Opaque, cut glass	>38,000,000

B_3: From 3-day broth culture of original
A_7: From 7-day agar culture of original

* All strains are antigenically *S. ogawa* serotype.

Summary

Considering even the inherent difficulties attending experimental cholera infections, it is apparent that progress has been slow in defining the factors of virulence of the pathogenic vibrios. In reviewing these problems, it is an inescapable conclusion that progress would be materially advanced if the mercurial variability of *V. cholerae* were more widely recognized and if greater effort were made to avoid or control it in experimental studies. However, this variability also could be better exploited with controlled, one-step mutations for analysis of virulence factors by methods of proven success in other host-parasite systems.

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Part II. Special Problems of Host-Parasite Relationships

STUDIES ON ORGANISMS GROWN *IN VIVO* TO REVEAL THE BASES OF MICROBIAL PATHOGENICITY

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The bases of microbial pathogenicity are the metabolic processes and products of pathogenic organisms that, in contrast to nonpathogens, enables them to produce disease in a susceptible host. Our knowledge of these bases of pathogenicity in most instances is vague especially for those organisms that grow to large numbers in the host before producing symptoms of the disease. This paper is concerned with discussing one method that has revealed hitherto unknown aspects of the pathogenic mechanisms of some bacteria and that could be extended to other microbes.

The definitions and the general biological nature of the bases of pathogenicity will not be discussed at length, since this has been done elsewhere;[1-4] only three points are emphasized in relation to this paper. First, any peculiar facet of the pathogen's metabolism accompanying its growth in the host tissues may be important in virulence, especially the reasons for the preferential growth of certain pathogens in certain tissues. Second, products of the pathogen should show some evidence of aggressive activity or toxicity before being considered important in pathogenicity; they should be tested for ability to produce one or more of the following effects—enhancement of virulence of organisms in an appropriate host, interference with the phagocytosis of otherwise susceptible organisms, interference with the bactericidal action of sera and white cell extracts, prevention of the intracellular digestion of organisms by phagocytes or promotion of their growth in these cells, and the killing of animals or the production of other pathological effects of the disease. Classical serological tests, for example, precipitation or complement fixation, serve only to indicate the specificity of the compound. Finally, although many bacterial products are antigenic, only a few are immunogenic, that is, their antigenicity contributes to active immunity against the disease; it is these immunogenic products that are important since usually they also play a role in virulence.

The In Vivo Environment for Producing All the Factors Responsible for Pathogenicity

The compounds responsible for the aggressive, toxic, and immunogenic properties of pathogenic organisms are usually proteins, carbohydrates, and lipids, either existing free or present as mixed complexes, for example, lipoproteins and mucopolysaccharides. Similar compounds are present in nonpathogenic species. The general metabolic processes whereby bacteria derive energy and synthesize the main structure of proteins, carbohydrates, and other compounds are probably similar for all organisms—pathogenic and nonpathogenic—and are not significantly altered by a change in conditions of bacterial growth. The concern here, however, is with the small peculiarities in the molecular structure of an end product that make it toxic, aggressive, or immunogenic. These peculiarities are certainly controlled by the microbial genome, but their production is phenotypically determined, especially by the nutritional conditions. Hence, although minimal media are useful in exploring such general processes as growth, reproduction, and protein synthesis, optimal media are of paramount importance when interest centers on these specific end products having biological significance in virulence.

When a pathogenic organism grows in the tissues of a host and produces disease, we know—by definition—that all the compounds and processes involved in pathogenicity are being produced. Hence the particular nutritional conditions of the host tissues during the infection and disease are fully adequate for the pathogen as regards the formation of virulence factors. It must be emphasized here that the decisive nutritional conditions in the host tissues undergoing bacterial attack may be different from normal physiological conditions and are continually changing.[5] The pathogen may react in some way to these semipathological conditions and to the mobilization of the host defense mechanisms; in fact, some virulence factors may well be produced as a direct reaction to the defenses of the host. It is at present impossible to reproduce *in vitro* even the physiological conditions operative in the normal host, not to mention the conditions occurring during inflammation and tissue breakdown by bacterial products. Apart from the unknown influence of host metabolites and enzymes on the metabolism of the pathogen, the excellent gaseous exchange system of the mammalian body and its variation in inflammation would be difficult to reproduce. The peculiar nutritional and other environmental requirements of various pathogens that lead to their preferential attack on specific tissues in disease (for example, *Brucella abortus* in bovine cotyledon, *Corynebacterium diphtheriae* in the throat and tonsils, and *Neisseria gonorrhoeae* in the urinogenital tract) are at present not understood and certainly not reproducible *in vitro*. It will be seen with reference to the anthrax toxin that, paradoxically, the tissues of the infected host can protect a bacterial product from destruction by the parent bacterium.[4] It is also possible that some pathogenic substances are large molecular compounds from the host that have been modified in some way by the pathogen. If this were so, it would be very difficult to produce the particular virulence factors in a synthetic medium without the addition of plasma or tissue components. In view of these considerations, it would be surprising if the complex metabolic activity of the pathogens *in vivo* were not different from that *in vitro*.

The successive loss of virulence and change in morphology during the artificial cultivation of recently isolated pathogens indicates the magnitude of the probable difference between organisms grown *in vivo* and *in vitro*. In this context, the S-R variation of many bacteria[6] is too well known to need description. The fact that the virulence of an organism can frequently be increased by animal passage also implies that substances and processes connected with pathogenicity are produced *in vivo* (possibly as a result of mutation or selection of virulent types) to a greater extent than *in vitro*. The work of Braun[7] on the effect of animal products on the virulence of bacterial populations has emphasized the effect of the *in vivo* environment on the virulence of bacteria.

Another effect that may be the result of a difference between bacterial products found *in vivo* and *in vitro* is the efficacy of living attenuated vaccines in cases where killed vaccines are relatively ineffective, for example, B.C.G. vaccine in tuberculosis and S.19 vaccine for contagious abortion. These attenuated vaccines appear to grow to some extent in the tissues of the host and persist for relatively long periods;[8-10] in this way they may have an opportunity to produce essential antigens at the most effective site. The killed vaccines may be ineffective because antigens originally present in the cultures were destroyed by the sterilization process. However the methods used for killing are usually relatively mild, and an alternative explanation is that the protective antigens are not present to any significant extent in the *in vitro* culture.

The rapid development of tissue culture techniques[11,12] with mammalian cells is allowing fundamental studies on such aspects as viral growth and multiplication under relatively convenient experimental conditions. Furthermore, certain intracellular bacteria have been grown in cells growing or maintained in tissue culture.[13,14] The success of tissue culture in producing certain immunogenic viral preparations makes the use of such techniques for problems of microbial pathogenicity tempting; so tempting, in fact, it is appropriate to the subject matter of this paper to emphasize that growth of a pathogen in tissue culture is still a long way from growth *in vivo* and that some aspects of pathogenicity may be missed in such studies. Thus most cells growing in tissue culture are quite different from their parent cells *in vivo* and this difference is often accompanied by a change in their susceptibility to the growth of certain viruses.[11,12] The pathogenicity of viruses changes in tissue culture.[11,12,15] Enders[16] has stated: "Just as repeated transfer *in vitro* may bring about alterations in the virulence or pathogenicity of bacteria, so serial passage of viruses in tissue culture has frequently led to changes in their capacity to induce disease when inoculated into a susceptible animal. Most often under these conditions a decrease in virulence has been noted. In some cases, however, especially when an agent is propagated in tissues derived from its natural host, an increase in pathogenic properties may follow." The attenuations during tissue culture of vaccinia virus for the rabbit,[17] yellow fever virus for man and monkey,[18] and certain poliomyelitis strains for monkeys or mice[11,15] are well-known examples of this phenomenon. Furthermore, in studies of intracellular bacteria within cells maintained in tissue culture the choice of cells may well be determined by technical considerations rather than their cogency to the infection *in vivo*. Monocytes are often used in such studies since they are relatively easily maintained for long periods whereas polymorphonuclear cells that play an important role in most infections are rarely used because, at the moment, they are difficult to maintain in culture.

It seems, therefore, when seeking substances responsible for pathogenicity, there is much to be said for studying organisms and their products obtained from infected animals. In recommending the use of organisms grown *in vivo* for studies on pathogenicity there is no suggestion of the existence of any absolute difference between them and those grown *in vitro* of the kind postulated by Bail and Weil.[19] Many virulence factors have indeed been recognized *in vitro*, for example, the capsular polysaccharides of *Streptococcus pneumoniae*,[20] just as many virulence factors can be formed by avirulent organisms. However, in the diseased animal a virulent organism is more likely to produce its whole armory of factors, whereas under conditions *in vitro* there is a distinct possibility of one or more being absent. Nor is it beyond the realm of possibility that, *in vitro*, compounds are formed that appear to be virulence factors but are never produced *in vivo*. Once the compounds responsible for pathogenicity and immunogenicity are recognized by studying organisms grown *in vivo*, the production of these compounds in defined media *in vitro* becomes possible and their chemical nature can be examined.

The Study of Organisms Growing In Vivo

The pathology of infection and disease. In order to understand the mechanisms of microbial pathogenicity and to design cogent biological tests for the bases of pathogenicity that can be used in subsequent fractionation of microbial products, some detailed knowledge is required of the pathology of the appropriate infection and disease. Unfortunately this knowledge is often sadly lacking. Only in a few cases (for example, *B. anthracis*[21] and *P. pestis*[22]) have studies been made of the pathogen and the reaction of the host to it during the first few hours of infection; these studies can indicate important aspects of the aggressive action and the type of compounds (such as antiphagocytic and bactericidal) to seek. Similarly—only recently and in a few cases[23]—has any precise knowledge supplemented the clinical pictures of the disease syndrome. In many chronic diseases and in those where a massive growth of the pathogen occurs before the host succumbs (for example, pneumonia) not only are the important toxic products of the pathogen unknown but also the exact nature of the fatal or other disease syndrome. Studies on anthrax[4] have shown how recognition of the former can follow from proper identification of the latter. The pathological syndromes in infectious diseases are difficult to study in detail due to the general technical problems of working with heavily infected animals (for example, the application of general safety precautions), the removal of bacteria from all fluids to be analyzed without affecting the composition of these fluids, and the assessing of any direct effect on any observation or determination (for example, blood sugar) of the metabolic activity of the growing organisms. One solution to these difficulties is indicated in studies on anthrax.[4] The infecting organism was allowed to grow in the host until the latter was about to succumb to the disease; then almost all the infecting organisms were removed from the host by antibiotic treatment in the terminal phases of the disease. This treatment does not save the host but effects a technically desirable removal of the organisms that, however, have had the opportunity of producing within the host their whole armory of harmful products.

Tests for the toxins of pathogens should relate as far as possible to the pathological effects of the infection, and any inconsistencies should be investigated. It will be seen later how an investigation, into the fact that the so-called toxin of *P. pestis* was not lethal for all the animal species killed by the infection, led to more complete preparations of the toxin(s) of this organism. Two factors (mucinase enzyme and lactic acid), in addition to the endotoxin, have been implicated in the pathogenesis of *Vibrio cholerae*[24] by use of a toxicity test with isolated rabbit intestine[25] rather than a simple lethality test with mice. The production of harmful substances different from endotoxins might be recognized for other gram-negative pathogens by using toxicity tests based on the different disease syndromes produced by these organisms.

The isolation of organisms grown in vivo and their products. Until recently there have been no studies either on the chemistry or on the metabolism of bacteria grown within the tissues of an infected mammalian host, and only one or two extracellular products present in exudates from infected animals[4] have received attention.

Using *Bacillus anthracis*, Smith *et al.*[26] developed a method for isolating pathogenic organisms from infected guinea pigs in sufficient quantity and of adequate purity to make possible a thorough examination of their chemistry and metabolism. Large quantities of host body fluids that contain products excreted by the pathogen were also obtained. Guinea pigs (800 to 1000 gm.) were infected intraperitoneally and intrapulmonarily with *B. anthracis*. A few minutes after death, the thoracic and peritoneal exudates were collected and mixed. By a three-step differential centrifugation at 0° C., the body fluids and saline-washed bacteria were separated from blood cells. One and one-half to 2 gm. of dry *B. anthracis* and 1½ to 2½ l. of body fluids containing the extracellular products of the organism were obtained from 100 guinea pigs; the bacteria were almost free from blood cells.

This method is applicable to infections in which the causal organism is extracellular and can grow freely in the blood or in exudates. Thus, in a small-scale trial,[26] organisms of the following species were readily obtained from infected guinea pigs: *S. pyogenes*, *S. pneumoniae*, *Listerella monocytogenes*, and *Staphylococcus aureus*. Recently Smith *et al.*[27] have used the method for the routine collection of *P. pestis* grown *in vivo*. Large numbers of organisms were found in the thoracic and peritoneal exudates and both body cavities were washed thoroughly with buffered saline to provide the maximum numbers of organisms. Approximately 1½ gm. of dried *P. pestis* organisms was obtained from 100 guinea pigs.

In the isolation of organisms that are intracellular parasites such as those producing leprosy, tuberculosis, and brucellosis, the organisms must be freed from their intimate relation with tissue cells. Hanks[28] and Gray[29] isolated *Mycobacterium lepraemurium* from *in vivo* sources. Infected testicular tissue was thoroughly homogenized at 0° C. in 0.2 *M* sucrose solution. After removal of coarse tissue by slow centrifugation, the bacilli were separated from tissue components by two centrifugations through dense solutions of sucrose and KCl that tended to support and remove the tissue components.

Segal and Bloch[30] have recently described a method for separating *M. tuberculosis* from the lungs of moribund mice. The infected tissue was homogenized at high speed in isotonic sucrose (0.25 *M*) solutions containing bovine albumin (0.25 per cent w/v) and the bacteria were separated from tissue components by differential centrifugation at 0° C. in this medium. The bacterial sediment was shown to be adequately free from tissue contamination by microscopical examination and by a comparison of the optical density, dry weight, and viable counts of suspensions with those of a similar suspension of *M. tuberculosis* grown *in vitro*. Relatively large quantities of organisms were obtained (4 mg.

per infected mouse lungs). J. H. Pearce, R. Fuller, J. Keppie and H. Smith (unpublished observations) have used a modification of this method for separating *Brucella abortus* from infected bovine foetal cotyledons. Approximately 1½ gm. of dried *Br. abortus* was obtained from one animal. Approximately 70 per cent of the bacteria originally present in the tissue were recovered but in a deposit that still contained tissue debris (approximately 3 parts debris to 1 part bacteria). Further purification of this deposit has not been carried out since interest has focussed on soluble bacterial products found in the large quantities of infected fetal fluids (to be discussed later).

It is important to point out a defect of the methods described above for preparing organisms grown *in vivo* for studies on pathogenicity. All the methods of isolation are designed for organisms present in the host at a late stage of the disease for the simple reason that a maximum yield of bacteria is desired. It is conceivable, however, that bacteria vary in their possession of different virulence factors throughout the course of an infection and, for the full picture, organisms should be studied at an earlier stage of the disease. The behavior of the pathogen in the first few hours after the primary lodgement, at least in the skin, is of paramount importance in the eventual outcome of the disease. It is obvious, however, that any study of the pathogen *in vivo* and its repercussion on the host at this stage in the infection must rely on microtechniques.

Chemical studies on organisms and their products produced in vivo. Organisms separated by one of the methods described can be used directly for studies on their metabolism[29,30] or extracted for chemical examination. The extraction technique should if possible indicate the location of a particular product in the intact organism so that it can be related to the pathology of infection and disease. If there is evidence of extensive lysis of the organism in the primary lesion or later in the disease, then of course components inside the bacterial cell may be of importance in pathogenicity. When there is no demonstrable evidence of extensive lysis, for example, in infections with *B. anthracis*, it seems that an aggressin or toxin must either be excreted or be present on the surface of the organism to have an effective role in pathogenicity. In this respect, it is important to make a thorough examination of the washings of the isolated bacteria for the presence of some easily removed surface component. The preferential removal of capsular material or the formation of cell wall preparations should always be attempted before an extract of the whole organism is prepared. If this proves unavoidable, phase-contrast microscopy as used by Tomscik[31] to detect precipitation patterns in capsules and in cell walls after the addition of appropriate antiserum may be a valuable aid in deciding whether a compound isolated from an extract of the whole organism was a component of the capsule or cell wall.

The extracellular products or easily removed surface components of the organisms are present in infected body fluids or tissue extracts that can be filtered through thin cellulose acetate filters having low adsorbtive capacity. For several reasons the bacterial products in these host fluids or tissue extracts will prove more difficult to identify than those compounds in the separated bacteria. First, there is the general difficulty of isolating small quantities of bacterial products from the large volume of host constituents. Second, unless the product is specifically precipitated or its biological activity specifically neutralized by homologous antisera it is not certain that the product is of bacterial origin; fortunately, in many cases the serological evidence is forthcoming. The third and major difficulty is the possibility of the production of antibody in the donor animals before the collection of the bacteria and their products. Compounds subsequently isolated from such materials might be complexes of antigen and antibody in antigen excess. This is unlikely to happen in acute experimental infections such as those occurring after intraperitoneal and intrapulmonary injection of *B. anthracis* or *P. pestis*, when the animal dies in 2 to 3 days. However, in chronic infections such as brucellosis or tuberculosis, these antigen-antibody reactions may be a very real problem. A possible solution might be to grow the organism in fetuses or in eggs where no antibody is produced to complicate the issue.

In view of these difficulties, if the important product of the pathogen appears from studies *in vivo* to be an extracellular or easily removed surface component, direct fractionation of the host fluids for the compound might be a less efficient way of finally identifying the compound than the following procedure. Attempts should be made to produce from a culture of the pathogen in a relatively simple medium *in vitro* a filtrate with the same important biological properties as the infected host fluids. If this can be done subsequent fractionation of the active compound should prove relatively easy. This might lead to a better fractionation of the host fluids and to a comparison between the final products from *in vitro* and *in vivo* sources.

Examples of the Use of Pathogens In Vivo for Studies on Pathogenicity

Work on these pathogens is described here and in each case the description is preceded by a summary of the problems that prompted the use of organisms grown *in vivo*.

Bacillus anthracis. Studies on *B. anthracis* grown *in vitro* had shed little light on the compounds and processes responsible for its pathogenicity. Bail and Weil[19] had clearly demonstrated the aggressive activity of *B. anthracis*, but in almost half of one century only scanty knowledge had accumulated about the chemical basis for it. Almost nothing was known about the killing power of *B. anthracis*. Neither the general nature of the lethal effect on the host nor the products responsible for it were known. In particular, no lethal endo- or exotoxin had been found in artificial cultures of the organisms, and no study of the chemical basis of the lethal aspect of pathogenicity had been possible.

B. anthracis was the first pathogen to be examined by Smith and his colleagues using organisms grown *in vivo*. Their studies of anthrax in the guinea pig have been reviewed in detail elsewhere;[4] only the salient points are summarized here, together with the results of some recent work.

(1) Previous hypotheses that death from anthrax was caused by the massive terminal bacteremia that led to blocked capillaries or to a deficiency of essential nutrients or other causes were proved wrong. Removal of the bacteremia with antibiotic did not prevent death and, in fact, death was determined when the bacteremia was still only 1/300th of its maximum. Hence a toxin was probably responsible for death.

(2) The fatal syndrome occurring during the last 10 hours of anthrax in the guinea pig was identified as secondary shock by extensive chemical and physiological determinations comparable to those used to define shock in other injuries.

(3) Shock was due to the extensive edema in anthrax. The anthrax toxin was found in the plasma of guinea pigs dying of anthrax as the result of a search of the tissues of such animals for an edema-producing toxin. In retrospect, it is now obvious that the small tissue-damaging activity of extracts from anthrax lesions that had been noted by a number of workers in the past but that had not been connected with the dramatic death by infection was due to traces of this toxin.

(4) This extracellular toxin that was lethal for mice and guinea pigs (dying in secondary shock), was neutralized by anthrax antisera, and was not connected with the capsule of *B. anthracis* or the lecithinase of this organism and *Bacillus cereus*.

(5) The toxin consisted of two practically nontoxic components: factors I and II that acted synergistically.

(6) After being recognised *in vivo* the toxin was produced *in vitro* in a medium containing serum.

(7) The toxin was immunogenic and connected with a nontoxic extracellular immunizing antigen that had been produced in a semisynthetic medium *in vitro*. An antiserum prepared against the antigen would neutralize the toxin and a purified preparation of this antigen replaced factor II in synergistic mixtures with factor I. Recently it was shown that the cultures in the semisynthetic medium also contained the other component of the anthrax toxin.[32]

(8) Factors I and II of the anthrax toxin were considerably purified by direct fractionation of the toxic plasma but the final preparations were contaminated with normal constituents of guinea pig plasma.[33] Factor I produced in the semisynthetic medium has been purified until it showed no evidence of heterogeneity on electrophoresis and ultracentrifugation and contained not more than approximately 6 per cent impurities detectable by a serological method.[34,35] It was a protein containing some carbohydrate residues. It was not powerfully immunogenic, but preliminary experiments suggest it enhances the strong immunizing activity of the preparation containing factor II.

The best preparation of factor II of the anthrax toxin was the purified immunizing antigen of *B. anthracis*[36,37] that, however, appeared to be a mixture of two serological components. This preparation was a protein.

The original recognition of the anthrax toxin in guinea pigs' plasma is recalled by the following phenomenon in toxicity tests with mixtures of the preparations of factors I and II purified from cultures *in vitro*. The edema-producing activity and the lethality for mice of these mixtures are enhanced by mixing them with normal sera of several animal species.[34] At present, the function of the serum in these tests is unknown but it may protect the toxin from a toxin-destroying system.

(9) The aggressins produced by *B. anthracis* are the extracellular toxin that appears to harm phagocytes and the capsular polyglutamic acid that appears to be antiopsonic.

(10) Attempts were made to study the metabolism of *B. anthracis* while

growing in the blood of guinea pigs; the uptake of amino acids was followed as were the effects of antimetabolites on growth and toxin production.

Pasteurella pestis. The study of *P. pestis* grown *in vivo* has recently thrown light on some of the problems of pathogenesis and immunity in plague.

Burrows and his colleagues[22,38,39] showed that virulent and avirulent strains of *P. pestis* that were indistinguishable by any test *in vitro* could easily be recognized by their behavior in the peritoneum of a mouse. The avirulent strain was phagocytosed progressively until all the bacteria disappeared. The virulent strain was also phagocytosed initially, but within half of one hour some organisms had become phagocytosis-resistant; these multiplied and killed the host. Fukui *et al.*[40] have shown that *P. pestis* behaves in guinea pig lungs exactly as it did in Burrows' experiments. After an initial lag period, virulent but not avirulent cells became resistant to clearance by the lung macrophages. Recently Cavanaugh and Randall[41] found that *P. pestis* became resistant to phagocytosis by polymorphonuclear leukocytes after a period within monocytes. Hence resistance to phagocytosis by polymorphonuclear leukocytes appears to be a major aspect of the aggressive action of *P. pestis in vivo*. The chemical basis for this resistance, which for most strains *in vivo* is accompanied by capsulation, was not clear. Fraction I or some complex containing it probably contributes to the phagocytosis-resistance of capsulated organisms since free Fraction I has an antiphagocytic action;[42] but in view of the work of Burrows and Bacon[38,39] compounds containing Fraction I do not appear to be the sole antiphagocytic substances. These workers prepared in a simple medium *in vitro*, phagocytosis-resistant *P. pestis* with no visible capsule from a number of mutants, including one apparently incapable of producing Fraction I.[43] The resistance—acquired by all virulent strains—was associated with the presence in the culture liquid of two precipitinogens V and W. These components were detected serologically in diffusion plates, but their direct responsibility for resistance to phagocytosis has not been proven. The culture filtrate containing them did not inhibit the action of phagocytes, whereas a phagocytosis-inhibiting substance was produced *in vivo*[22] and by *in vitro* culture of virulent *P. pestis* in mouse peritoneal fluid or whole blood.[38]

Recently, Smith *et al.*[27] have begun a study of *P. pestis* and its products from infected guinea pigs to clarify these problems on the aggressive action of *P. pestis* and the following anomalies in studies of its toxicity and immunogenicity.

A puzzling feature of previous studies on the toxicity of *P. pestis* was the lack of relationship between the susceptibility of guinea pigs to the infection and to the so-called toxin.[44,45] Although this toxin was lethal for mice it was not so for guinea pigs,[45] whereas the two hosts were equally susceptible to plague infection.[44,46] This anomaly suggested that in plague either guinea pigs are killed by a toxin different from that killing mice, or the toxin, previously isolated, is a degraded form of a native toxin that kills both species. To remove the anomaly it was essential to obtain a preparation from *P. pestis* that would kill guinea pigs, and such material seemed most likely to be found in organisms (and their extracellular products) obtained from guinea pigs dying of plague.

In vaccination studies on plague, a similar anomalous situation existed indicating that the currently accepted chemical basis for immunity to plague was as insecure as that for toxicity. It was generally accepted that live attenuated vaccines immunized guinea pigs as well as mice, but dead vaccines (without artificial adjuvant) were relatively ineffective for guinea pigs although adequate for mice.[47] Therefore, either the essential immunogen for guinea pigs was different from that for mice and found only during the growth *in vivo* of living vaccines, or there was a common protective antigen for both species that was so degraded during the preparation of vaccines as to be capable only of protecting mice. There is evidence for these suggestions in previous work on the fractionation of *P. pestis* for the protective antigen(s). The preparation that has been claimed as the principal antigen for immunizing against plague is Fraction I, a nontoxic component of the envelope obtained from saline extracts of acetone-dried *P. pestis*.[48] However, even in mice Fraction I as isolated did not account for more than a small proportion of the protective activity of dead *P. pestis*,[48] and therefore the possibility of Fraction I being one of a number of protective antigens or a fragment of the native antigen was at once apparent. In guinea pigs, Fraction I, as with dead bacteria, had little effect unless injected with an adjuvant. The "residue antigen" (the material left after saline extraction) had some protective effect for guinea pigs as well as for mice and appeared to contain a residual amount of the native antigen(s). In view of the shortcomings of the above preparations, organisms grown *in vivo* were investigated for protective antigens that would immunize guinea pigs without adjuvants and mice, more effectively than isolated Fraction I.

Studies[27,49,50] on *P. pestis* grown *in vivo* were first directed to finding whether hitherto unknown extracellular products comparable to the anthrax toxin were responsible for the toxicity and immunogenicity of this pathogen for guinea pigs. However, plasma and body fluids collected rapidly after the death of guinea pigs from plague and immediately freed from organisms had insignificant immunizing or toxic properties, although they interfered with the phagocytosis of *P. pestis* by polymorphonuclear leukocytes of guinea pigs and mice. Attention then turned to the organisms isolated from infected guinea pigs.

Whole organisms grown *in vivo* were toxic for guinea pigs and mice when injected with streptomycin into animals that were treated with streptomycin before and during the toxicity test; dead animals proved uninfected with *P. pestis*. The numbers of streptomycin-killed organisms needed to kill guinea pigs (LD_{50} approximately 2×10^{10}) and mice (LD_{50} approximately 1×10^{8}) were far less than those found in guinea pigs (6 to 16×10^{10}) and mice (5 to 7×10^{9}) when they died of plague. Toxemic animals died with the clinical signs of shock as did infected animals, but the formation of edema by the products (see below) was not sufficiently large to indicate that this was the primary cause of shock.

Hence *P. pestis* grown *in vivo* contained toxin(s) that killed guinea pigs as well as mice, and the yield of toxin was such as to provide a reasonable explanation for the death of both species as a result of infection, provided the toxin was liberated *in vivo*. This explanation for death would hold only if the liberated toxin(s) was immediately fixed to tissue since only a trace of free toxin could be detected in freshly collected body fluids.

The nature of the native toxin(s) is still unknown. When the organisms were disrupted with ultrasonic waves, the toxicity for guinea pigs and mice was found both in the soluble extract (approximately 70 per cent of the organism) and in the residue (approximately 30 per cent of the organism). The latter was largely cell wall material, contained no intact organisms, and could be retained in solution at *p*H 9.2 after a short treatment with alkali.

The toxicity for guinea pigs found in the soluble extract was about twice that found in the residue. Both preparations were far less toxic than the whole organisms, and the sum of the separate toxicities did not account for the toxicity of the whole organisms. This anomaly was explained by the fact that toxicity for guinea pigs appeared to be due to the synergistic action of two components. Fractionation of the extract on diethyl-aminoethyl cellulose columns separated the two components, one of which could be replaced by a solution of the residue.

The toxicity for mice was predominantly in the extract while some (approximately 10 per cent) remained with the residue. Furthermore the mouse toxin was 2 to 4 times more potent when liberated in the ultrasonic extract than when fixed in the intact organisms. When fractionated on diethylamine ethyl cellulose and hydroxylapatite, a preparation was obtained at least as potent as the previous preparations of the mouse toxin but different from them in possessing a residual toxicity for guinea pigs, for example, it had a greater ability to produce edema in the skin of guinea pigs.

The possibilities that the mouse toxin was identical or connected with one of the two components of the guinea pig toxin and that all these materials were incomplete portions of a larger native toxin occurring in the intact organism will be investigated in future work.

We turn now to the immunogenic activity of the extract and residue prepared from *P. pestis* grown *in vivo* by treatment with ultrasonic waves. A solution of the residue immunized guinea pigs effectively and accounted for the immunizing activity in this species of intact *P. pestis* isolated directly from infected guinea pigs and killed in three different ways.[50] This residue also immunized mice against infection with *P. pestis* but was less active in this respect than the material dissolved by the action of ultrasonic waves that largely accounted for the activity in this species of the original organisms; the extract did not immunize guinea pigs.

The immunizing activity of the residue or the extract for mice could not be explained by their estimated content of Fraction I. The latter injected without adjuvant produced no significant protection of guinea pigs.

The residue, immunogenic for guinea pigs and mice, contained lipid, carbohydrate (including the lipopolysaccharide of Davies[51]), and protein (including "combined" Fraction I) but no nucleic acid. It was suggested that this residue was an important part of a cell wall complex of *P. pestis* to which Fraction I (and possibly the virulence antigens) were attached firmly near the cell wall and loosely in the body of the envelope. This cell wall complex with the attached envelope could be regarded as the best antigenic material for the protection of all species. If it was split by autolysis, acetone drying and, to a lesser extent, by washing or treatment with ultrasonic waves, then the fragments lost protective power. This loss might be more apparent in some species than others. Thus the residue from ultrasonic treatment contained a relatively large fragment that retained high immunogenicity for guinea pigs but only a low one for mice. The extract contained smaller fragments—ineffective for guinea pigs—but collectively more effective for immunizing mice than one of them, namely, Fraction I. In fact it appeared that Fraction I was an ill-defined fragment of a more complete antigen. Thus whole organisms and various products therefrom had greater immunizing activity for mice than was warranted by their content of Fraction I. Furthermore, this antigen was originally isolated in two forms, Fraction IA and IB; IA, but not IB, contained carbohydrate and, if anything, IA had a greater immunizing activity for mice than IB.[48]

It is not yet known whether the residue left after extraction of *P. pestis* with ultrasonic waves is a single complex since it has been examined only in solution after treatment with alkali. This treatment did not destroy the immunogenicity for guinea pigs, but it was relatively drastic and may have hydrolyzed some linkages. However, the solution of the residue thus obtained revealed no gross heterogeneity in the ultracentrifuge and showed only two components in the electrophoresis apparatus. On serological precipitation in gel diffusion plates, against unabsorbed rabbit antiserum to live *P. pestis*, a concentrated solution of the residue formed only three lines in contrast to the multiplicity of lines formed by the soluble extract or whole organisms.

Hence the study of *P. pestis* grown in infected guinea pigs has revealed the compounds responsible for toxicity and immunogenicity in this species and has questioned the validity of Fraction I being, per se, the basis of immunity in mice. With regard to the aggressive action of *P. pestis*, both the extract and the residue enhanced the virulence of *P. pestis* for guinea pigs and interfered with the phagocytosis of *P. pestis* by mouse and guinea pig phagocytes. Chemical fractionation will identify the nature of these biologically active compounds. It appears at present that the compounds responsible for immunogenicity in guinea pigs are not the same as those responsible for toxicity. Thus the residue did not contain as much of the toxicity of the original organisms as did the extract, which, however, failed to protect guinea pigs.

As a result of this work on *P. pestis* grown *in vivo*, similar toxic and immunogenic products have been obtained from *P. pestis* grown *in vitro*. The immunogenic materials from this source may form the basis of an improved nonliving vaccine against plague.

Brucella. In spite of intensive research with organisms grown *in vitro*, the compounds and processes responsible for the all-important intracellular growth of this pathogenic species are unknown and the most effective vaccination procedures demand live attenuated vaccines.[9,10,52] The study of the chemical basis of pathogenicity of this species and of immunity to brucellosis using organisms grown in tissue culture,[13,14] in eggs,[53] and *in vivo* may be fruitful fields of research.

Recently, a team of workers in our laboratory (unpublished observations) have been studying *Br. abortus* in cattle. Preliminary results of their work are as follows. At the abortion of a pregnant cow experimentally infected with virulent *Br. abortus* (strain 544), 80 to 85 per cent of the total organisms in the mother and fetus are in the fetal placentae. In seeking an explanation for this preferential growth of *Br. abortus* in fetal placental tissue, extracts of this tissue have been shown to be nutritionally more complete for the growth of *Br. abortus in vitro* than extracts of other tissues. The reasons for this are being investigated. Extracts of *Br. abortus* isolated from infected bovine placental tissue (see above) and the filtered fetal fluids have been examined in biological tests side by side with similar extracts of *Br. abortus* and filtrates from cultures *in vitro*. *Br. abortus* produces in filtrates substances, probably easily removed surface components, that are immunogenic to guinea pigs and mice, appear to promote the growth of *Br. abortus* in the white cells of bovine blood, interfere with the bactericidal action of normal cow serum on *Br. abortus*, and produce delayed hypersensitivity. Further work should indicate the nature and interrelationship of the substances responsible for these different biological activities and their importance in the pathogenicity of *Br. abortus*.

It is hoped that work summarized here on three pathogens and other experiments on *M. tuberculosis* and *Streptococci* reviewed elsewhere[4] will stimulate work on other pathogens, for example, *Staphylococci*, using organisms grown *in vivo*.

Acknowledgments

I am indebted to D. W. Henderson, F. R. S., for his interest and to J. Keppie and my other colleagues at the Microbiological Research Establishment for their contributions to this work.

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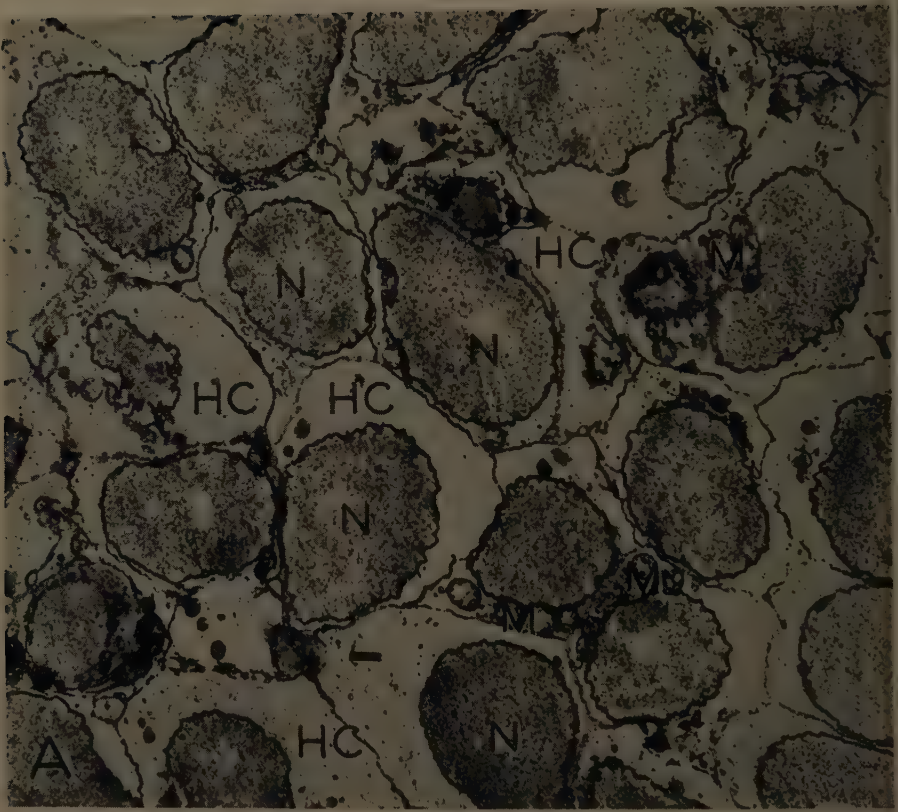
Discussion of the Paper

Robert P. Williams (*Baylor University College of Medicine, Houston, Texas*): Smith's suggestion that one take a closer look at the pathology of infectious diseases has been initiated almost literally in our laboratory. I. L. Roth and I have been investigating the host-parasite relationship in anthrax at the submicroscopic level by examining ultrathin sections of infected mouse spleen in the electron microscope. Mice were sacrificed when moribund, and blocks of spleen tissue were immediately fixed in potassium permanganate. After fixation and dehydration, the blocks were embedded in araldite epoxy resin. This procedure resulted in good preservation of bacterial cells and host tissue so that the ultramicroscopic features of both could be examined simultaneously. Ultrathin sections were cut and examined in the electron microscope.

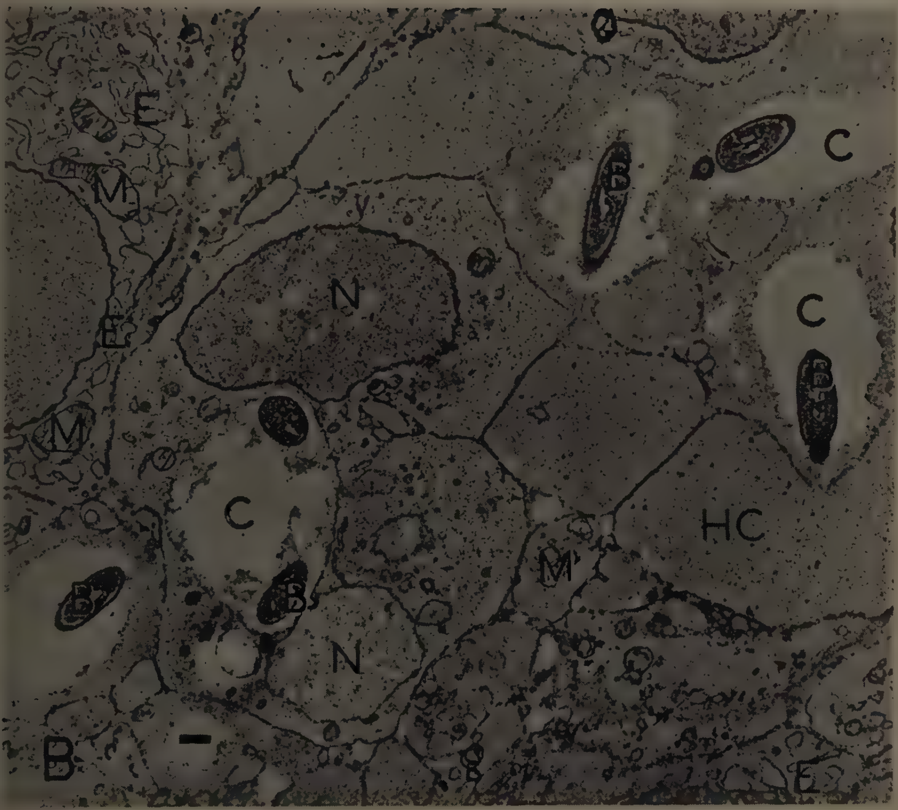
Bacterial cell structures were well preserved and included the cell wall, plasma membrane, nuclear elements, capsular material, and apparent prespore structures. The appearance of these bacterial structures in host tissue was not at all different from that reported for ultrathin sections of *Bacillus* organisms cultured *in vitro*. Host tissue cells showed well-preserved cytoplasmic and nuclear membranes, mitochondria, and endoplasmic reticulum. However, there was a marked difference between normal and infected tissue, particularly in the areas surrounding bacteria. Tissue damage in these areas was extensive. Complete dissolution of host structure was observed immediately adjacent to the bacteria. Cytoplasmic membranes appeared to be disrupted, and nuclear, mitochondrial, and endoplasmic reticulum elements were absent from the cells. The cytoplasm of these host cells appeared almost homogeneous and finely granular in contrast to normal cytoplasm that was distributed heterogeneously within the cell and appeared coarsely granular. Host cells farther removed from the bacteria retained more of their structure, but showed apparent vacuolation and disruption of mitochondria, homogeneous granulation of the cytoplasm and, in some cases, an increase in endoplasmic reticulum. The latter observation is interesting in view of the importance of the endoplasmic reticulum in protein synthesis and suggests that the host cells were responding to the bacterial invasion by increased protein synthesis.

Immediately surrounding each bacterium was a clear area devoid of any visible structure. We have designated this zone as a "cytopathogenic area." Whether the area is of bacterial or host origin we do not know. It might represent a lacuna left by the disappearance of the bacterial capsule during fixation and embedding of the specimen.

We believe the damage to host tissue produced by the bacteria is due to the anthrax exotoxin that was first demonstrated by Smith and his colleagues. We are continuing our investigations in an effort to determine whether injection of purified anthrax exotoxin will produce similar host cell damage, and also to study the progressive changes brought about by infection upon host cell structures. Figures 1A and B (on pages 1228 and 1229) are low-power electron micrographs of normal and infected mouse spleen. Figure 1B demonstrates the damage brought about by the bacterial infection.



FIGURES 1A and B. A, electron micrograph of normal mouse spleen; B, mouse spleen infected with *B. anthracis*. Both pictures taken at a magnification of ×2600. B, bacteria;



C, cytopathogenic area; E, endoplasmic reticulum; HC, host cytoplasm; M, mitochondria; and N, host cell nucleus.

MODIFIED SERUM RESISTANCE OF BACTERIA FOLLOWING INTRACELLULAR RESIDENCE*

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In the course of studies on the multiplication of *Brucella abortus* within monocytes, we have observed an interesting example of differences between *in vivo* and *in vitro* grown bacteria. Our observations involve a phenotypic modification of certain bacterial properties that can occur following intracellular residence. Since the modification affects the bacteria's resistance to bactericidal serum factors, it furnishes not only an additional example for potential phenotypic differences of bacteria growing *in vitro* and *in vivo*, but it also supplies an interesting model for interactions that may occur between the host's humoral and cellular defense systems.

The methods employed for maintaining monocytes *in vitro*, the conditions permitting ingestion of brucellae by the phagocytes, and the procedure for permitting and assessing intracellular multiplication of the ingested brucellae, without concurrent opportunity for extracellular multiplication, have been described previously.[1] With these methods we have observed: (1) significant intracellular multiplication of smooth virulent brucellae in monocytes from susceptible hosts, such as normal guinea pigs; (2) lesser or insignificant intracellular multiplication of these bacteria in monocytes from more resistant brucella-infected guinea pigs, or from normal naturally resistant rats; and (3) an absence of intracellular multiplication in all types of monocytes tested whenever the bacteria involved belonged to rough, relatively avirulent strains.[1-3] The data to be described here are concerned only with smooth, virulent brucellae and with monocytes harvested, after intraperitoneal injection of saline, either from normal or previously brucella-infected guinea pigs.

The tests on the susceptibility to serum of brucellae harvested from monocytes were prompted by several considerations: (1) the lack of a detectable dramatic destruction‡ of brucellae within mononuclear phagocytes derived from infected and normal guinea pigs; (2) the seeming lack of correlation between resistance to brucellosis and brucellacidal serum factors revealed by tests with slant-grown brucellae; and (3) the possibility that a serum-susceptibility test might serve as a sensitive tool for detecting differences between small numbers of intracellularly-grown and slant-grown sister cells. It was considered likely that intracellular residence of brucellae might result in subtle changes, perhaps of cell wall components, that might affect the subsequent

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‡ Dramatic destruction is meant to imply the type frequently seen following phagocytosis of nonpathogenic bacteria or of opsonized pathogenic bacteria that are not normally intracellular parasites.

survival of the organisms in the presence of humoral factors. Initial observation suggested that this was indeed the case. Following residence in monocytes from normal guinea pigs, increased resistance of S brucellae to bactericidal bovine serum was noted in comparative tests with slant-grown brucellae; in contrast, there appeared to be an increased susceptibility to serum following residence in monocytes from guinea pigs infected with *B. abortus* six months previously. Whereas the first stated alteration was confirmed in a large series of subsequent tests, additional study of the latter effect, that is, the increased susceptibility, has so far yielded only equivocal yet suggestive results. This will be discussed in more detail later on.

TABLE 1

BACTERICIDAL SYSTEM

	Bactericidal test (ml.)	Control (ml.)
Buffered beef extract broth (BBE)	0.1	0.1
Normal bovine serum	1.7	0.0
Saline	0.0	1.7
Bacterial inoculum	0.2	0.2
Total (ml.)	2.0	2.0

Aliquots are removed after 3 and 6 hours and plated on modified tryptose agar.

TABLE 2

SURVIVAL OF *BRUCELLA ABORTUS*, SA-S, IN THE BACTERICIDAL SYSTEM

Inoculum	Hours of incubation	
	3	6
Slant-grown bacteria	23* (19–27)	12 (9–16)
Monocyte-grown bacteria	76 (72–79)	28 (24–32)

* Per cent survival of original inoculum. Figures in parentheses are approximate 95 per cent confidence limits.[4]

TABLE 1 shows the bactericidal system we have employed. Inocula were obtained either from 48-hour-old modified tryptose agar slants or from infected monocytes broken up with tiny glass beads. So-called normal bovine serum was obtained from local slaughter houses; such sera had agglutinin titers of 1:20 or less and lost their bactericidal activity following heating or specific absorption. BBE-supplementation was used in all of our bactericidal tests because saline alone is brucellacidal whereas 5 per cent BBE in saline supports survival and slow multiplication of brucellae while permitting bactericidal effects of serum. TABLE 2 shows the extent of increase in serum resistance of brucellae harvested from monocytes of normal guinea pigs 72 hours after initiation of ingestion. It will be noted that 3 and 6 hours after introduction of these monocyte-grown brucellae into the bactericidal system, there is appreciable survival. In contrast, similar brucellae grown on modified tryptose agar for 48 hours and then exposed to serum show much less survival.

Table 2 also illustrates the slow rate of killing in the brucellacidal serum system, a fact that sets it apart, at least from the standpoint of kinetics, from those typical for many other Gram-negative bacteria.[5] When this slow-killing process was studied in more detail, that is at hourly intervals, it became apparent that the differences illustrated in table 2 represent principally a postponement of the onset of killing of bacteria harvested from normal monocytes. However, once detectable bactericidal activity had commenced, the rate of decrease in viable count was similar for slant-grown and monocyte-grown bacteria. Since the initiation of bactericidal serum effects against monocyte-grown brucellae occurs with such a delay, it was considered possible that return to typical serum sensitivity might be preceded by bacterial multiplication. Tests indicated that this was indeed the case. As illustrated in figure 1, with incubation in a growth medium for 4 hours after harvest from monocytes, the sensitivity of the brucellae to serum returns to the same level that is typical for slant-grown brucellae, and this period coincides with the time estimated to be required for a doubling of the bacterial population, that is, in the average one division per original bacterium. The estimate of the amount of multiplication is based on both the increase occurring during preincubation for various periods of time after harvest (figures within the circles) and that occurring in the diluted broth controls after the beginning of the bactericidal tests. The

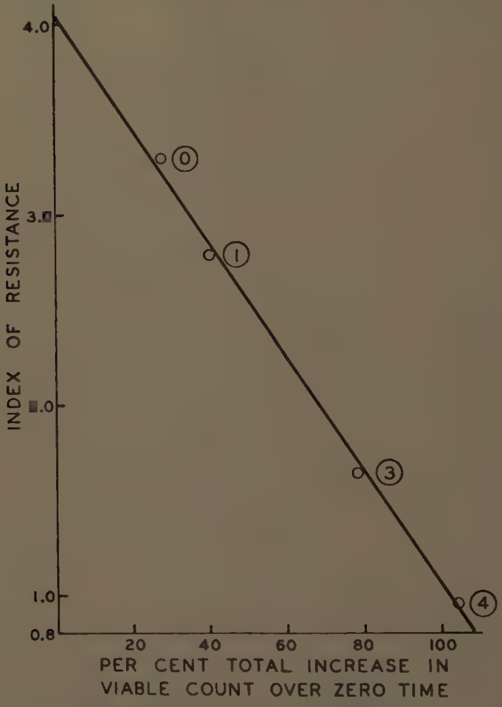

Figure 1. Resistance to bovine serum of monocyte-grown *B. abortus* as function of total increase in viable count during preincubation and bactericidal test.

index of resistance is the percentage survival of the monocyte-grown brucellae after 3 hours' exposure to bovine serum divided by the percentage survival of the slant-grown organisms after the same period of time. These data thus indicate that the alteration in serum resistance following intracellular residence is phenotypic and not genotypic and is lost after a bacterium has had an opportunity to divide once in an extracellular environment. If division is delayed by decreased temperature following harvesting from monocytes, the loss of the increased serum resistance is also delayed.

Populations of monocyte-grown brucellae display a significantly slower increase in viable counts in 5 per cent BBE-saline than slant-grown brucellae of the same genotype. It therefore appeared possible that part of the increase in resistance to serum might be associated with this alteration in growth dynamics. This appeared particularly likely since a striking relationship between susceptibility to serum and metabolism has been demonstrated for certain other Gram-negative bacteria in recent studies by Michael and Braun.[5] However, as illustrated in TABLE 3, no correlation appears to exist in the case of *B. abortus* between multiplication, presumably reflecting rates of metabolism, and susceptibility to serum. Also, in contrast to tests with *Shigella flexneri* and *Escherichia coli*,[5] in other experiments the susceptibility of *B. abortus* to bactericidal serum was not altered by the presence of bacteriostatic levels of chloramphenicol. Therefore, the demonstrated modification of resistance to serum after intracellular residence of brucellae may involve basic changes that affect both growth and serum susceptibility that at least in this organism do not have to be causally related.

TABLE 3

TESTS FOR POSSIBLE RELATIONSHIPS BETWEEN MODIFICATION OF GROWTH AND SERUM SUSCEPTIBILITY OF *BRUCELLA ABORTUS*

Treatment	Per cent survival after incubation in serum + BBE		Per cent increase after incubation in saline + BBE	
	3 hours	6 hours	3 hours	6 hours
Prior growth on				
Modified tryptose agar (2 days)	59	19	128	190
Gerhardt-Wilson agar (2 days)	15	8	98	121
Prior growth on modified tryptose agar (2 days) at				
37° C.		52*		194
32° C.		50		240
Room temperature		51		274

* The bovine serum used in this experiment was less active in inactivating *B. abortus* than other sera tested.

The possibility of an involvement of misleading artifacts in the described alteration of serum resistance has been considered. At least two potential sources of error come to mind: (1) possible coating of the monocyte-harvested brucellae with constituents of the mononuclear cells that, in turn, might protect the brucellae from serum bactericidins; and (2) a possible retention of significant numbers of intact brucella-containing monocytes that, although lysed late during serum exposure, may initially lead to protective effects. Several observations minimize the importance of the first point. Thus it has been shown that monocyte-harvested brucellae still adsorb brucellaphage[6] as efficiently as their slant-grown sister cells; also, both monocyte- and slant-grown brucellae react with specific antibody in the same manner, leading to a blocking of bactericidal action of normal serum. In addition, incubation in products of infected or noninfected monocytes did not increase resistance. The second point, that is, a possible involvement of initially unlysed monocytes, is unlikely because direct microscopic observations reveal few unbroken monocytes containing bacteria and because filtration through filter paper, which will remove almost all intact mononuclear cells, does not alter the observed resistance patterns (TABLE 4).

TABLE 4
SERUM RESISTANCE OF FILTERED AND UNFILTERED BACTERIAL HARVEST FROM INFECTED MONONUCLEAR CELLS

Harvest	Per cent survival in bactericidal system		
	3 hours	6 hours	9 hours
Filtered*	88	49	35
Unfiltered	86	51	40

* Filtration through Whatman No. 2 paper, known to remove more than 95 per cent of intact monocytes.

TABLE 5
RESISTANCE TO BOVINE SERUM OF *BRUCELLA ABORTUS* GROWN IN MONONUCLEAR CELLS FROM INFECTED AND NONINFECTED GUINEA PIGS

Brucellae for bactericidal test	Average viable count of these culture harvest (per ml.)	Per cent survival after 3 hours in bactericidal test system			
		Experiment No.			
		I	II	III	IV
Slant-grown		12	6	8	10
Monocyte-grown, from animals that are:					
Normal	7.3×10^5	—	63	60	33
Infected					
1 week*	6.2×10^5	77	59	54	22
4 weeks*	8.3×10^5	60	86	63	—
7 weeks	1.8×10^5	44	39	70	49
14 weeks	3.8×10^4	42	54	34	24

* Guinea pigs of strain different from the rest.

Tests also have been made on the extent of modified serum resistance following residence of brucellae in monocytes from guinea pigs infected with *B. abortus*, SA-S, 1 to 14 weeks earlier. As shown in TABLE 5, increased serum resistance still resulted. However, there is some fluctuation in the extent of this modification. There is a tendency for brucellae harvested from mononuclear phagocytes of guinea pigs infected 7 or more weeks to be more sensitive to normal bovine serum bactericidins than brucellae harvested from uninfected animals or animals infected for a shorter length of time. Whatever the reason for such apparent decreases in serum resistance, it may be suggested that such trends would be beneficial to the host in its interactions with the parasite. Present data do not suffice to establish definitive relationships between duration of infection in monocyte donors and extent of modification of serum resistance; yet, as indicated in FIGURE 2, there are suggestions of a correlation between extent of intracellular multiplication and extent of increased serum resistance.

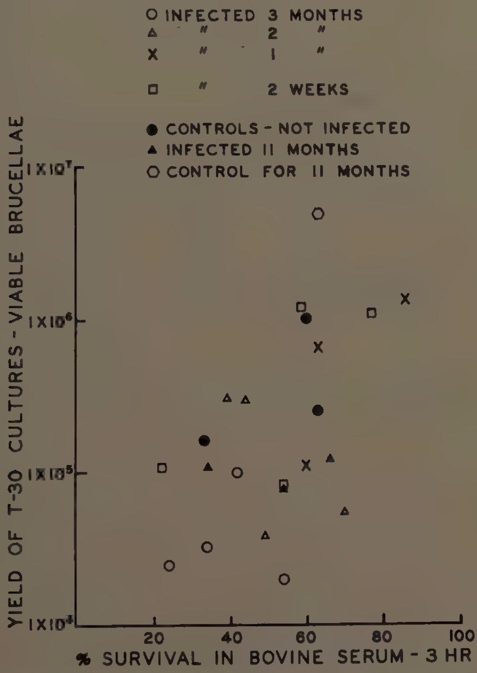

FIGURE 2. Correlation between intracellular growth and subsequent sensitivity to bactericidal factors of bovine serum.

The cause of the described increased serum resistance of brucellae following intracellular residence or multiplication is now under investigation. No readily apparent qualitative antigenic changes have been detectable so far. Quantitative rather than qualitative changes in certain wall components may be involved, and tests to determine this possibility are now under way.

In any event, the described observations indicate that the normal habitat of this intracellular parasite *B. abortus* may have a decisive influence on the future fate of the pathogen. Once the parasite has passed through the barrier posed by the normal bactericidal blood factors, it may become less susceptible to normal bactericidins as a consequence of intracellular growth. At a time when extracellular factors would be expected to be of prime importance in preventing spread of the brucellae from damaged infected cells to new host cells, phenotypic modification of the parasite can have led to an alteration of properties controlling host-parasite relationships in a manner favorable to the parasite. Subsequent interactions between host cells and parasite may lead to a reversal of such trends and serum bactericidins may again become of greater importance. Although the biochemical basis for the described modifications still requires elucidation, our data again demonstrate that proper growth conditions are required in order to detect and to determine the nature of biochemical factors influencing host-parasite interactions.

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NONSPECIFIC AND SPECIFIC CELLULAR REACTIONS TO INFECTIONS*

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Introduction

During the last few years it has become increasingly evident that the response to antigenic stimulation by bacterial components or bacterial infection is not limited to antibody production. A number of reaction mechanisms other than circulating antibodies are acquired by the host as a consequence of such stimulation. Thus functional versatility is added to existing humoral and cellular mechanisms representing the host's capacity to respond to stimulation. Some of these changes are specific for the agent used for induction, whereas others are nonspecific in character. Frequently alterations of the host's resistance to infection with antigenically related or unrelated pathogens are associated with these changes. Observations concerning alterations of nonspecific resistance to infection and the adaptive capacity of the RES under various conditions have been reviewed recently.[1,2] In view of such findings, a consideration of the host's total capacity to respond to primary and secondary infection has come within reach. Also, a separation into humoral and cellular reactions is desirable.

However, difficulties are encountered when alterations on a cellular level are considered. First, quantitative information is scarce, partially due to the lack of techniques allowing accurate measurements with single cells. Second, it is extremely difficult, if not impossible, to obtain homogeneous populations of cells that belong to a widely distributed system such as the RES without introducing procedures that by themselves act as nonspecific or specific stimuli. Third, analysis of experimental results is hindered by the fact that the contributions of the extracellular environment cannot be excluded either in *in vitro* or *in vivo* experimentation. In the following some cellular reactions induced by infection with tubercle bacilli are reported.

Review of In Vitro Experiments Concerned with "Immune Monocytes" in Tuberculosis

As a facultative intracellular parasite,[3] the tubercle bacillus enters with mononuclear phagocytes (MN) an interrelationship of long duration. For this reason interest is focused on possible changes of this interaction during primary infection with virulent tubercle bacilli or as a consequence of immunization with BCG. Observations derived from *in vitro* studies are summarized briefly (TABLE 1). In most experiments it is noted that cells from immunized animals exert some restriction upon intracellularly located bacilli. This effect is either expressed as inhibition of intracellular multiplication or as increased resistance of the cells to the accumulating bacilli. In either case further para-

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sitization of uninfected cells is prevented or retarded. It is of interest that in all experiments in which retardation of multiplication was observed the ratio of bacteria to cells was kept low for the period of phagocytosis (approximately 1:1), and that only a small fraction of the MN had ingested bacilli. From the data published thus far, it is not possible to decide whether this initial numerical relationship is critical. However, experiments with MN derived from desensitized vaccinated donors indicate that the restrictive capacity of MN for bacilli is observed under these conditions and that the destructive effect of the bacilli on cells is less with cells derived from desensitized donors or with very small inocula.[10] Existence of cross immunity was not investigated except for Elberg's experiments.[9] In these, reciprocal immunity was observed when MN derived from animals immunized with *Mycobacteria* or *Brucella* were tested with either one. However, the presence of serum from an infected animal was essential for the expression of immunity in the culture system. This serum effect proved to be nonspecific and equally observable with specifically absorbed sera. It appears possible that the pretreatment with trypsin of the MN prior to infection removed some component from the cellular surface essential for the expression of the immune effect; this material could possibly be reintroduced into the system with "immune" serum. This view is substantiated by the recent discovery of cytophilic antibodies arising during immunization that exhibit great affinity for monocytes[11] and by the fact that in other experiments without

TABLE 1

IN VITRO STUDIES CONCERNED WITH "CELLULAR" IMMUNITY

Author (reference)	Animal	Multiplicity		Mixture		Outcome of interaction
		Bac:MN	Phagocytosis (%)	MN	Serum	
Lurie[4]	Rabbit	0.1:1	—*	Immune	Normal Immune	Restriction of multiplication
Suter[5]	Guinea pig	1:1	5–10	Immune	Normal Immune	Restriction of multiplication
Mackaness[6]	Rabbit	—	50–70	Immune	Normal	Uninhibited multiplication
Berthrong[7]	Guinea pig	>10:1	40–60	Immune	Normal	Inhibition of intracellular accumulation of bacilli
Abe[8]	Guinea pig	2:1	10	Immune	Normal Immune	Inhibited intracellular multiplication Inhibited intra- and extracellular multiplication
Elberg[9]	Rabbit —	10:1	—	Immune	Normal Immune	No effect Decreased cellular destruction

* Not recorded in references.

the use of trypsin this need for immune serum was not observed. It is important to note that findings with *Brucella* almost parallel those obtained with *Mycobacteria*. Thus MN derived from guinea pigs immunized with an attenuated strain are capable of suppressing intracellular multiplication of virulent *Brucella*.[12] Pertinent data obtained with other models are reviewed elsewhere.[9]

Transfer of Protective Effect by Cells from Immunized Donor to Normal Recipients

The importance of cellular changes induced by immunization or primary infection for the manifestation of resistance against secondary infection is conjectured from observations obtained in *in vivo* studies, but direct proof is not available. As in the case of delayed hypersensitivity, transfer experiments with cells should give direct information on the importance of cellular factors for the immune response in tuberculosis. Experiments were done using mice and guinea pigs. The prospective donors were vaccinated with attenuated *Mycobacteria* strains BCG or R_1R_v. Cells were collected either from lymph nodes, the peritoneal cavity, or spleens. They were washed and resuspended in balanced salt solution, counted, and were injected intraperitoneally into recipient animals. Recipients received 2 to 5 consecutive injections amounting to a total of 50 to 70 $\times$ 10^6 cells per animal. Different experiments were done for the assessment of successful transfer of protective immunity against infection with tubercle bacilli. They are reviewed briefly in the following (details will be published elsewhere).

Transfer experiment 1: interaction in vitro between tubercle bacilli and MN from recipient guinea pigs. Guinea pigs were injected intraperitoneally or intracardially with lymph node cells from donors previously vaccinated with BCG. Peritoneal exudate MN were collected, infected with a small number of tubercle bacilli (strain R_1R_v), and were cultured on glass slides.[3] Enumeration of the intracellular bacillary population at the beginning and after 5 and 8 days of incubation showed that over this period of time the increase of intracellular bacilli was less in MN derived from recipients of immune cells and vaccinated animals than in cells from normal controls (TABLE 2). MN from recipients of normal cells exhibited no inhibitory power toward intracellular organisms. No attempt was made to determine whether the cells recovered from recipients were a mixture of the animal's own cells with donor cells.

Transfer experiment 2: extent of skin lesions in guinea pigs after intradermal injection of attenuated Mycobacteria. The severity of skin lesions in guinea pigs induced by intradermal injection of tubercle bacilli can serve as an indicator of differences of pathogenicity between strains of tubercle bacilli.[13] Guinea pigs that had been injected intraperitoneally with saline or MN from normal or BCG-vaccinated donors were challenged intradermally with graded dilutions of BCG. The sizes of the developing and healing skin lesions were measured (FIGURE 1). It was quite apparent that recipients of immune cells developed smaller lesions that appeared later and began to heal earlier than in controls.

Transfer experiment 3: extent of pulmonary lesions and their content of stainable bacilli after intravenous infection of mice with strain $H_{37}R_v$. Intravenous injection of mice with moderate numbers of virulent tubercle bacilli can result in chronic infection with pulmonary involvement. Extent and severity of pulmonary lesions and the number of tubercle bacilli contained can serve as a measure of the relative resistance of the animals.[14] Four groups of 10 mice were used: one served as controls, one was vaccinated with BCG, and two received repeated injections of immune cells (4 times and 2 times, respectively).

TABLE 2

TRANSFER EXPERIMENT 1: MULTIPLICATION OF TUBERCLE BACILLI (STRAIN R_1R_v) WITHIN CULTURED MN FROM NORMAL, BCG-VACCINATED, AND RECIPIENT* GUINEA PIGS

Source of MN	Average number of bacilli per 100 MN counted		
	1†	5	8
Donor: normal	2.4	7.1	11.1
immunized	2.3	2.8	2.9
Recipient (I.P.): normal	3.0	6.8	8.3
immunized	2.6	3.5	4.1
Recipient (I.C.): normal	2.3	3.6	10.7
immunized	2.5	3.5	4.1

* "Recipients" were injected intraperitoneally or intravenously with lymph node cells from donors previously infected with BCG 5 days prior to collection of MN for the cultures.

† Days of cultivation.

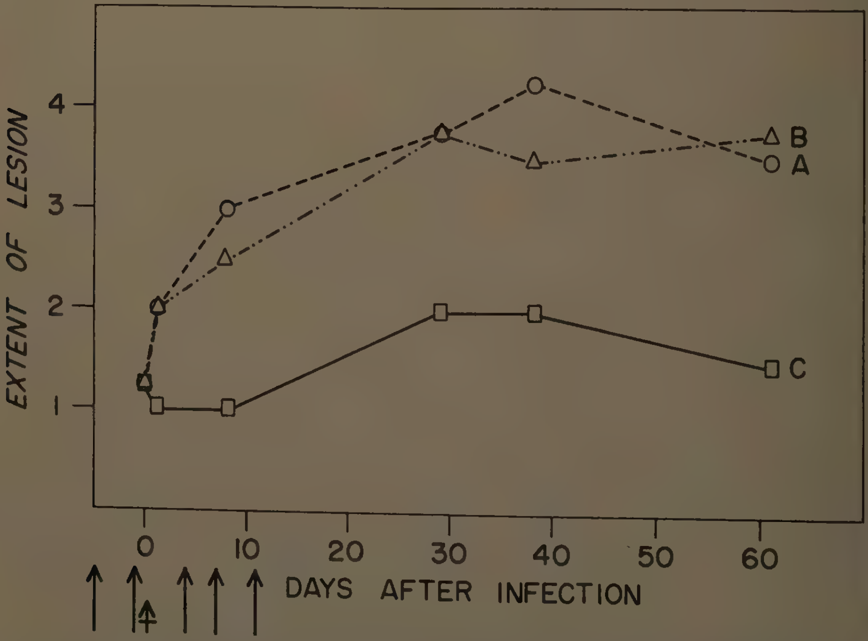

FIGURE 1. Transfer experiment 2. The extent of skin lesions in arbitrary units in guinea pigs after intradermal injection of 0.1 ml. of undiluted culture of BCG. Animals of group A received saline, of group B cells from normal donors, and of group C cells from vaccinated donors. The points on the curve are averages of readings done on two animals in each group. ‡ = intradermal injection; ↑ = transfers of cells.

All animals were infected intravenously with $H_{37}R_v$ and killed 5 weeks later. A summary of blindfolded scores of sections of the lungs for lesions and stainable bacilli (TABLE 3) reveals that BCG and recipient animals had fewer bacilli in their lungs than had the control animals. Determination of the number of microscopic fields that had to be searched at random to detect 100 cells containing stainable tubercle bacilli proved to be the most sensitive criterion. Blindfolded scoring of the sections of the lungs for extent and severity of lesions revealed a difference between control animals and the BCG-vaccinated and recipient groups.

Transfer experiment 4: mortality of mice infected with strain Vallée. Inoculation with a relatively large number of highly virulent *Mycobacteria* causes an infection of relatively short duration, terminating fatally in approximately 18 to 25 days. Upon challenge with such a virulent infection no difference was found between control group and animals that had received repeated injections of immune cells. Mice previously vaccinated with BCG survived significantly longer than the other groups.

TABLE 3

TRANSFER EXPERIMENT 3: EXTENT AND SEVERITY OF LUNG LESIONS AND THEIR CONTENT IN TUBERCLE BACILLI IN MICE INFECTED WITH TUBERCLE BACILLI (STRAIN $H_{37}R_v$)

Group	Lung scoring	Average No. of bacilli per 100 cells counted	No. fields counted*	No. bacilli per field counted
Control: saline	100	4.5	103	4.4
BCG-vaccinated	73	2.0	2546	0.08
Recipient:† 4 transfers	79	3.0	383	0.78
Recipient: 2 transfers		3.7	335	1.1

* The number of microscopic fields searched to find 100 cells infected with acid-fast bacilli.

† "Recipients" were injected intraperitoneally 4 or 2 times with exudate MN from previously immunized mice. Injections were done at intervals of 3 days. Challenge infection after the second transfer.

These experiments show quite clearly that transfer of some protection against challenge infection can be achieved by injections of mononuclear cells (lymph node cells, mononuclear exudate cells, and spleen cells). These results are in agreement with recent findings by Sever.[15] However, in our experiments protection was limited to challenge that causes only chronic infection. The implication is that MN of animals immunized by an attenuated infection acquire a property that can endow increased resistance in a recipient animal. It is not possible even to guess whether the transferred cells themselves are directly involved in the immune response to the challenge infection in the recipient or whether they carry a factor or factors that are released in the recipient. That a cellular mechanism is involved is indicated not only by the failure of immune serum to transfer protection[15] but also by the fact that MN of the recipient animal acquire some power to inhibit intracellular multiplication of tubercle bacilli *in vitro*. Any speculation on the nature of such a transfer factor is based on scant information. A transfer factor was described in the case of delayed hypersensitivity in man,[16] and an apparent α-L-globulin could be extracted from monocytes from tuberculin-sensitive guinea pigs but not from normal ones.[17] Experiments done in our laboratory indicate that exudate monocytes from sensitized guinea pigs release a "transfer factor" when cultured *in vitro* for a period of 5 days (unpublished observations). The nature of this factor is not yet known. Although these experiments suggest that some cellular factor contributes to acquired immunity in experimental tuberculosis, the possibility that the transferred cells from immunized donors produce some antibody in the recipient animal should be considered. The cells could possibly continue to shed antibody after transfer, or they could initiate a secondary response upon challenge infection of the recipient animal.

Metabolic Differences between Normal Phagocytes and Phagocytes from Infected Animals

Possible alterations occurring within phagocytes after infection are manifold. First, these cells could acquire some antibody or antibodylike molecule with immunological specificity for the infective agent. Second, metabolic changes could occur that are not necessarily specific for the pathogen. Changes of the second type have been found in human as well as in experimental infections expressed as either gains or losses of metabolic functions. The presence of histochemically detectable acid phosphatase in macrophages in the granulomatous lesion induced by tubercle bacilli, and its absence from normal histiocytes or monocytes are important in this respect.[18] When a comparison was made of the rate of oxygen uptake by polymorphonuclear leukocytes from normal and tuberculous guinea pigs, a significant difference was found.[19] Exudate leukocytes from infected animals exhibited a higher rate of oxygen uptake than those from normal animals. The magnitude of the increase was a function of the virulence of the strain used for infection (FIGURE 2). In more recent studies, a quantitative determination of acid phosphatase in MN cultured from normal and BCG-vaccinated rabbits was undertaken. Exudate MN from rabbits immunized 12 weeks earlier and from controls were maintained in large T-flasks in balanced salt solution containing rabbit and calf serum. Survival of cells during the period of cultivation was measured by counting those cells that remained attached to the glass upon replacing the maintenance medium. After three to five days of cultivation, the cells were removed from the glass, counted, and their content of acid phosphatase determined. The cells were disrupted in a sonic oscillator, and the lysate was incubated in the appropriate medium with paranitrophenyl phosphate as substrate. The amount of paranitrophenyl produced was determined spectrophotometrically and compared with readings obtained from standard solutions. Some of the cultures were infected with living or dead tubercle bacilli 24 hours prior to the determinations (TABLE 4). Cells derived from BCG-infected rabbits contained a significantly higher amount of enzyme than cells from uninfected controls. Infection of the immune cells *in vitro* resulted in a decrease of activity, whereas normal cells responded with an increase. This response was independent of the number of surviving cells upon infection.

These observations dealing with metabolic reactions to infection indicate that primary infection can induce alterations on a cellular level. These reactions appear to be independent of the original stimulus as far as their specificity is concerned. As with respiration, the metabolic function measured has no apparent specific relation to the inducer, namely the tuberculous reaction. In the case of acid phosphatase it is conceivable that infection with tubercle bacilli introduces materials rich in esters of phosphoric acid. However, a number of stimuli can elicit the presence of this enzyme in macrophages, such as cultivation *in vitro*[20] or the injection into tissues of materials that cause granulomatous reactions.[21] These observations then suggest that during in-

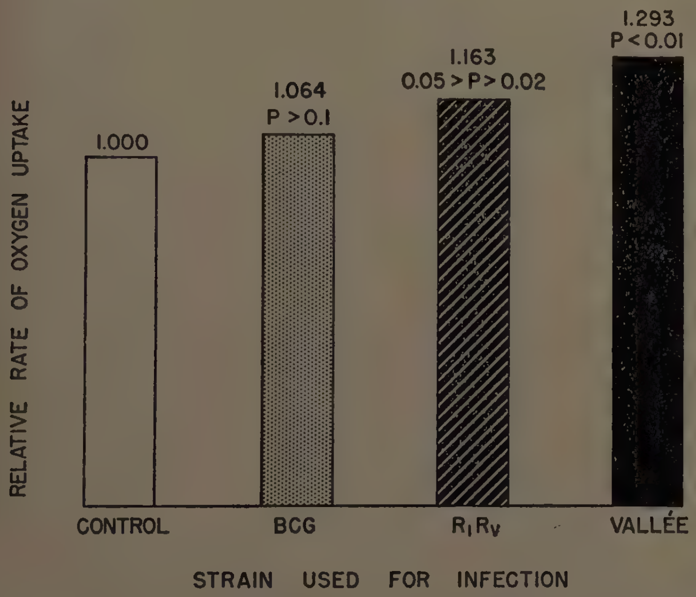

Figure 2. Relative oxygen uptake rates of leukocyte suspensions rich in PMN from normal guinea pigs and from guinea pigs infected with tubercle bacilli. The oxygen uptake rates were calculated as μl./100 μg. cellular phosphorus an hour. The absolute value for PEC from normal animals is 36.5 ± 1.9 μl., O_2/100 μg. phosphorus an hour. The number of animals to which each bar refers are as follows: control, 31; BCG infected, 15; R_1R_v infected, 8; Vallée infected, 6. With the exudate from each animal, at least 2 determinations were obtained. Reproduced by permission of the *Journal of Experimental Medicine*.[19]

Table 4
Acid Phosphatase Activity of MN Cultured from Normal and BCG-infected Rabbits

Rabbits		MN cultures			
Treatment	Number	Number	Infection	Surviving cells (%)	Acid phosphatase activity*
None	5	51	None BCG	100 46	33 ± 10 57 ± 11
BCG	4	30	None BCG	100 125	60 ± 13 40 ± 4

* 10^{-6} *M* *p*NP per 10^6 cells.

fection cellular alterations occur that are part of a general reaction pattern to stimulation rather than a specific response to the agent involved. This does not preclude the fact that the reactions themselves are highly specific. It remains to be studied whether these changes contribute to the increase of resistance to homologous or heterologous infection and whether they are involved in the suppressive effect exerted by cells upon bacteria *in vitro* or *in vivo*.

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Discussion of the Paper

M. B. Lurie (*Phipps Institute, University of Pennsylvania, Philadelphia, Pa.*): I have two points to make at this time:

While there is no question as to the importance and analytic advantages that may be derived from studies of host-parasite interactions of isolated mononuclear phagocytes grown *in vitro* as compared with such endeavors in the whole organism, with the infinite complexities that obtain *in vivo* the obvious stricture should be borne in mind that, unless the *in vitro* conditions simulate the essential requisites of the former, tissue culture studies throw no significant light on the host-parasite relationships as they occur in the body. A case in point is the work of Elberg *et al.*,[1a] who found that virulent tubercle bacilli ingested by mononuclear phagocytes derived from normal animals and cultured *in vitro* kill the macrophages, whereas cells derived from immune animals, in the presence of a specific or nonspecific immune serum, are not killed by these bacilli. These data are significant in themselves as they relate to the *in vitro* system analyzed, but in relation to the host-parasite relationships *in vivo* they are merely laboratory experiments. *In vivo* macrophages of normal animals, especially during the second week after a primary infection and before allergic sensitivity develops, often swarm with tubercle bacilli, yet these cells show no injury whatsoever. Therefore the death of such parasitized cells under the given *in vitro* conditions has no bearing on the behavior of such cells *in vivo*.

The second point I commend to your attention is the similarity of the *in vivo* effects on the host-parasite relationships between BCG immunization on the one hand and induced hyperthyroidism on the other. Both suppress the accumulation of tubercle bacilli within the macrophages. Hsu and Kapral[2] have apparently confirmed the bacteriostatic effects of hyperthyroidism with guinea pig monocytes cultured *in vitro*. However, hyperthyroidism hastens the mortality of mice with acute tuberculosis. This is probably due to the increased sensitivity of hyperthyroid mice to endotoxins, as shown by Melby and Spink.[3] It is noteworthy that BCG vaccination also increases the susceptibility of mice to endotoxin 100-fold, as demonstrated by Suter.[4] Furthermore, Howard *et al.*[5] found in addition that, while BCG markedly enhances the susceptibility of mice to endotoxin, the reticuloendothelial cells of these same mice possess increased bacteriostatic capacity against the very *Salmonella typhimurium* to the endotoxin of which they are markedly more sensitive than normal mice. A parallel is thus established between the effects of BCG vaccination and hyperthyroidism on the activation of the RES on the one hand and on the increased sensitivity to endotoxin on the other.

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IRRADIATION, IMMUNIZATION, AND SENSITIVITY TO TOXIC SUBSTANCES: CONSIDERATIONS IN A CONCEPT OF CELLULAR IMMUNITY

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Introduction

The idea that cells, by virtue of their structure and composition, may be or become immune to particular agents has found expression in various concepts of cellular immunity. The resistance exhibited by vertebrates, invertebrates, and plants has, in many cases, been related to particular cells.[1] Natural and tissue immunity are usually explained on biochemical and genetic bases. However, those individuals interested in antibodies and their formation have in some cases accounted for local resistance in terms of local antibody production.[2] The pharmacologic concept of tolerance also has been ascribed to resistance of individual cells to particular drugs.[3] To workers concerned with the development of bacteriophage (particularly with bacteria in the lysogenic state), cellular immunity means the resistance of a particular bacterial cell to superinfection while latently infected with phage.[4] Recent studies with mammalian viruses in tissue culture indicate that cells may exhibit resistance to infection with a virus to which they previously had been exposed.[5] Finally, however, there is an area of research in which the term cellular immunity has come to be best known and understood. This is the area that deals with phagocytic cells that are believed to acquire resistance or an enhanced capacity for destruction of a bacterial agent following previous exposure to it or its products. That such cells are actively involved in resistance to disease has been emphasized by recent reports of Sever[6] and Suter[7] involving quantitative transfer of resistance to tuberculosis via monocytes. Most of the work in this area has been concerned with studies of the tubercle bacillus in isolated mononuclear cells from guinea pigs and rabbits.[8-12] Other studies have concentrated on the host-parasite relationship between phagocytes and *Brucella*,[13,14] fungi,[15] *Salmonella*,[16] *Escherichia coli*,[17] and *Pseudomonas*.[18]

A detailed and critical analysis of this latter type of cellular immunity (that is, that dealing with a bacterium and a phagocytic cell) recently has been presented by Elberg.[12] It is this kind of cellular immunity with which I shall be concerned in this paper.

Radiation Studies

During the course of studies on the effect of radiation on the host-parasite relationship existing between *Pseudomonas* and mouse peritoneal phagocytes, it was observed that the bacteria could grow intracellularly and destroy cells of irradiated animals.[18,19] However, if the animals previously had been

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immunized with suspensions of heat-killed *Pseudomonas*, this effect of irradiation was lacking. That is, the organisms were destroyed in much the same fashion as in cells from nonirradiated animals. This relationship is illustrated in FIGURE 1.

It can be seen that, whereas few organisms survived four hours intracellularly in cells of normal or normal-immunized animals, many bacteria survived in cells from irradiated mice. There was some increased survival of bacteria in cells from immunized mice with higher doses of radiation, but the organisms were controlled by the cells. These findings seemed to us a true indication of cellular immunity since these studies were carried out in the complete absence of humoral substances. In fact, when specific antibody was added to preparations of cells from immunized and nonimmunized mice, no change was seen in the reaction other than an increase in the rate of phagocytosis. The ultimate multiplication or destruction of microorganisms was the same whether antibodies were present during phagocytosis. Similar findings have recently been reported by Jenkin *et al.*,[20] who used techniques different from ours. The interesting thing about this type of cellular immunity, of course, is that it is only apparent following irradiation. In cells from nonirradiated animals there was no greater efficiency of killing with cells from immunized than with cells from nonimmunized mice (FIGURE 2).

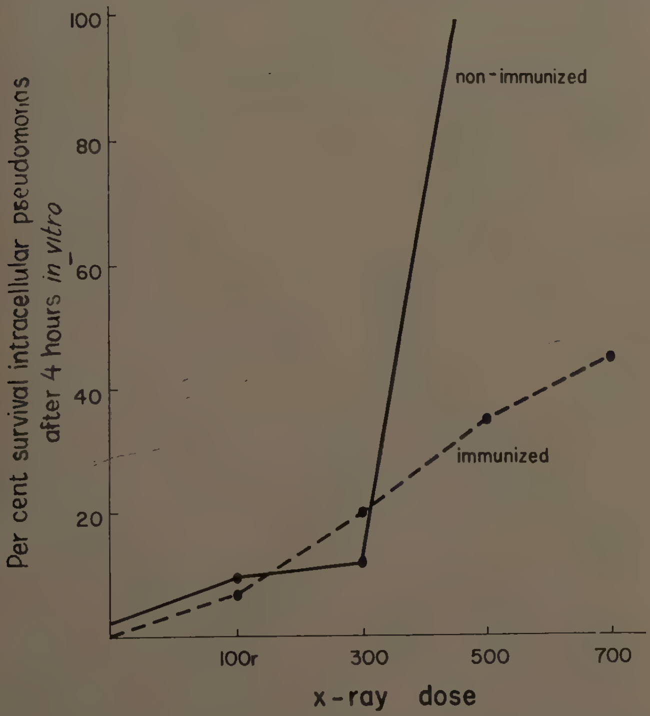

FIGURE 1. The intracellular survival of *Pseudomonas* in cells from irradiated mice.

In an attempt to explain the relationships between immunization and irradiation, certain other studies have been carried out that we feel support a concept of cellular immunity. Before proceeding, however, and in order to fully appreciate the interaction between cells, bacteria, and their environment, certain quantitative relationships must necessarily be established. The next few figures will, I believe, indicate these relationships.

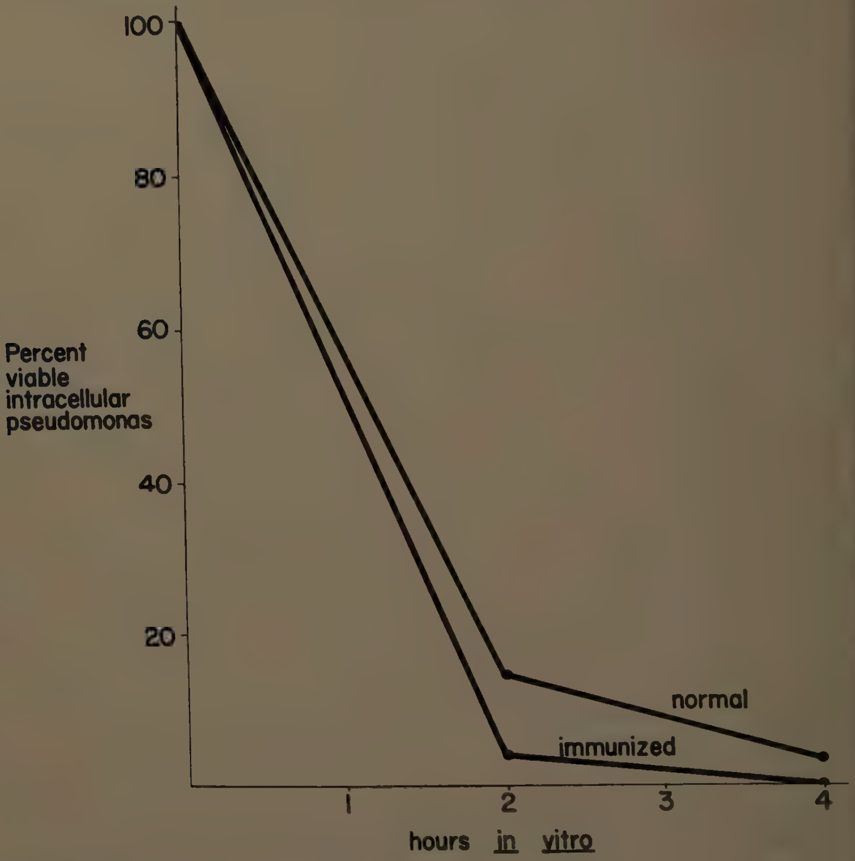

FIGURE 2. The killing of intracellular *Pseudomonas* by phagocytes from normal and immunized mice.

The data in FIGURE 3 indicate that the number of *Pseudomonas* organisms needed to kill an irradiated animal decreases with time postirradiation. That is, the LD_{100} (lethal dose, 0 per cent survival at 30 days) is not constant for irradiated animals. If mice receive 600 rad. of X radiation, then on successive days' postirradiation the number of organisms needed to kill them decreases from 10^8 to 10^4. If, as we believe, resistance to *Pseudomonas* is largely dependent upon destruction of the organism by cells and, if there is a minimal number of bacteria lethal for the mouse, this number should depend upon the ratio of organisms to cells.

Following irradiation, animals show a distinct leukopenia.[21] There is a decrease in the total number of phagocytic cells both in the peritoneal cavity (TABLE 1) and in the circulation. In addition, cell regeneration is inhibited as a consequence of irradiation. Therefore, with time postirradiation, a decrease in number of bacteria lethal for the mouse should be expected. A threshold level of bacteria determined by the number of available phagocytic cells should have to be exceeded in order for death to occur.

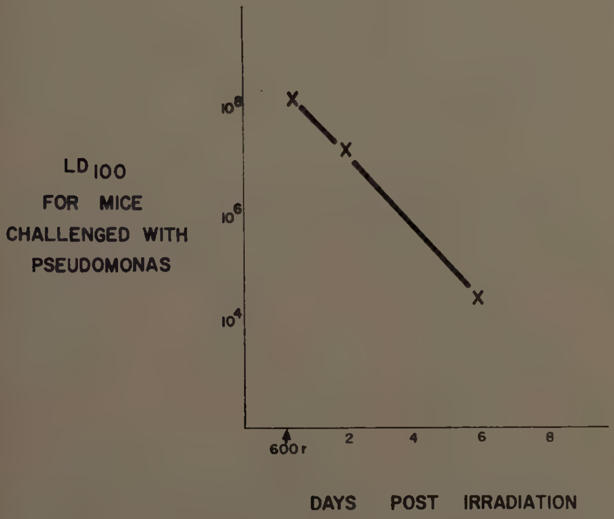

FIGURE 3. The lethal dose of *Pseudomonas* for irradiated mice.

TABLE 1
THE EFFECT OF IRRADIATION ON THE QUANTITY OF PERITONEAL EXUDATE RECOVERABLE FROM MICE

X-ray dose (rad.)	Average cell volume (mm.³)/mouse
0	3.4
100	3.0
300	2.5
500	1.2
700	0.1

FIGURE 4 indicates the relationship between the numbers of organisms required to kill normal and immunized mice. In two strains of normal mice (CF1 and Webster) the LD_{80} 30 days is approximately 4 × 10^8 *Pseudomonas* given intravenously. Typically there is an initial clearance of injected organisms that, in animals that die, is followed by an increase in the total number of bacteria up to 10^8 or more. Death occurs at approximately 24 hours. In immunized animals we find that the number of bacteria required to kill is increased by approximately 90 per cent. The question, of course, is why this increased resistance in immunized animals?

Figure 5 illustrates the decrease and subsequent increase in total bacterial count in irradiated animals that succumb to infection with *Pseudomonas*. The initial clearance is the same regardless of time postirradiation. However, it requires only a few hours for the bacteria to multiply to lethal numbers in animals injected 5 to 7 days after irradiation, whereas a period of days is needed for those injected immediately after irradiation. With normal animals given a lethal inoculum of *Pseudomonas* the curve is the same as for

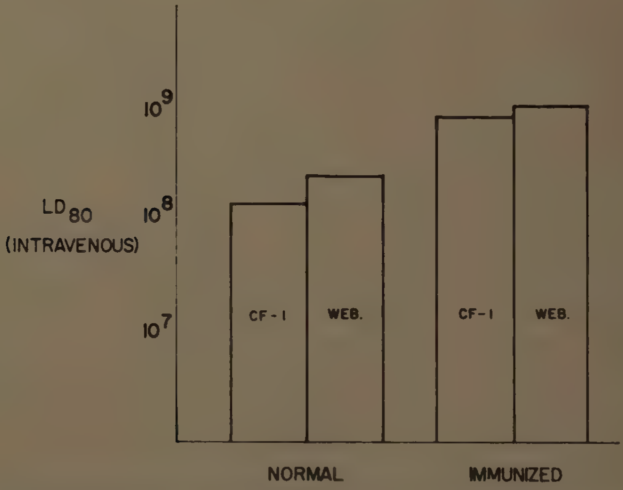

Figure 4. The lethal number of *Pseudomonas aeruginosa* for mice.

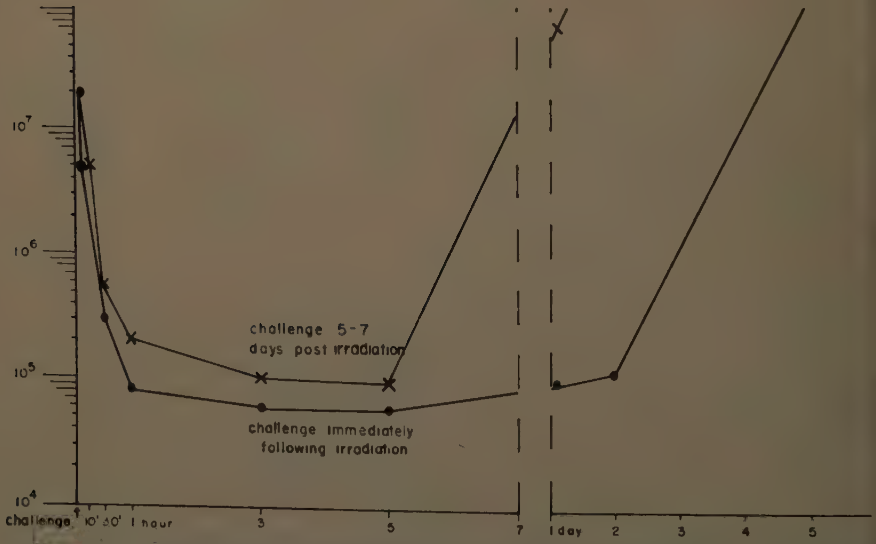

Figure 5. The number of *Pseudomonas* recovered from irradiated (600 rad.) mice challenged with *Pseudomonas* immediately and seven days following irradiation. (Each point represents the average of counts on at least five mice.)

animals injected 5 to 7 days postirradiation. This rather well-defined relationship between the lethal number of bacteria and the state of the animal as determined by time and dose of irradiation emphasizes the quantitative nature of *Pseudomonas* infection. We believe such data support the idea that death results when the balance between the number of bacteria and the number of phagocytes tips in favor of the parasite. Similar quantitative relationships between host and parasite have been shown to exist in experimental anthrax infection[22] and in experimental tuberculosis.[23] It has been possible to devise rather interesting experiments that exploit these relationships and at the same time confirm them.

We have shown that the reticuloendothelial system may be seeded with an intravenous injection of *Pseudomonas* followed immediately by a rather large dose of polymyxin.[24] Under these conditions the numbers of organisms in the blood are reduced by 90 per cent or more and practically all of the bacteria that survive may be found within the liver and spleen. We have used this procedure to establish intracellular organisms in mice. By maintaining an adequate level of antibiotic in the animals, it is possible to limit the infection to the intracellular environment. FIGURE 6 illustrates what happens under these conditions. A sublethal injection of 2×10^8 organisms was made intravenously into mice given 600 rad. X radiation and, in one group of mice, this was followed immediately by a similar injection of 1000 units of polymyxin. These animals were treated daily with the antibiotic for the first three days, during which period none died. The curves illustrate the cumulative number of deaths of animals in the several experimental groups. The first curve shows the normal time course for death in animals not treated with antibiotic. In the treated animals we assume the organisms were suppressed and deaths were withheld until such time as the drug was removed.

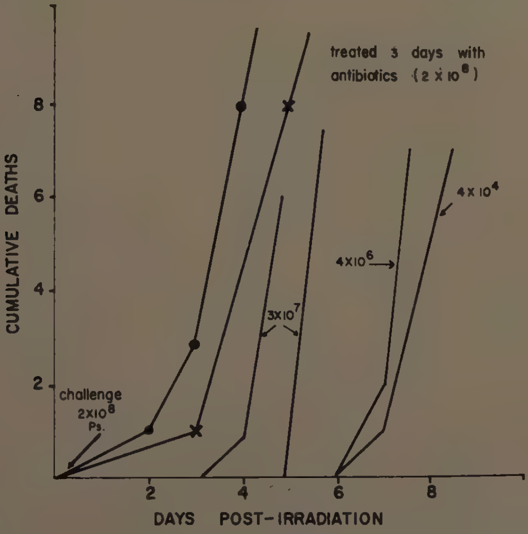

FIGURE 6. The number of *Pseudomonas* lethal for mice given 600 rad. X radiation.

At the time of removal the animals promptly died. In the other groups illustrated the animals were irradiated but not challenged until 5 and 6 days later. The challenge dose was considerably lower than that used immediately following irradiation, thus illustrating again the decreased resistance of irradiated animals during the period 5 to 7 days postirradiation. These data indicate how intracellular bacteria can be suppressed with drugs until therapy ceases. Then in the absence of such host factors as phagocytes they multiply and kill their host as if no treatment had been given. Without irradiation the animals are resistant. The time of challenge is unimportant once the cell count is down, let us say any time after the fourth day postirradiation. Also, fewer organisms are needed for challenge at this time.

In all our studies with intracellular *Pseudomonas* we insisted that phagocytosis occur *in vivo* under those conditions that naturally would obtain. Accordingly, mice received intraperitoneal injections of the organism approximately 16 hours after stimulation of the peritoneum with gelatin or warm mineral oil. Following a period of an hour or so, during which phagocytosis occurred, the peritoneal cells were aspirated, washed, and suspended in suitable maintenance media. With these techniques it was possible to prepare a suspension of cells that contained a given number of intracellular *Pseudomonas*. The infected cells were in turn used as inocula for other animals, that is, the challenge inoculum consisted of intracellular organisms rather than bacterial cultures.

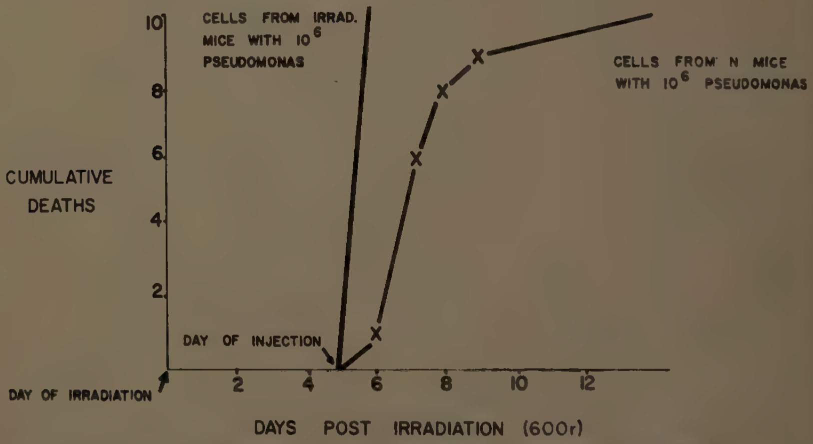

FIGURE 7. The infection of irradiated mice with intracellular *Pseudomonas*.

FIGURE 7 illustrates what happened when cells obtained from normal or from irradiated mice, each containing the same number of *Pseudomonas*, were used to challenge irradiated and nonirradiated animals. When cells from irradiated mice were used to challenge irradiated animals 6 days postirradiation, the animals promptly died. Apparently the organisms were released from cells and multiplied to lethal numbers. When infected cells from nonirradiated animals were used as inocula, the results were somewhat unexpected. It had been assumed that normal cells would be able to destroy the intracellular *Pseudomonas* and therefore no deaths should be expected among the animals receiving these cells. It is obvious that, although there was some delay in killing when such cells were used, nevertheless all of the challenged animals died. This suggests possibly that the milieu of the irradiated animal is not entirely suitable for maintenance of normal cells. Another possibility, of course, is that even a few organisms released from the transplanted cells would prove lethal to animals irradiated 4 to 6 days previously.

To recapitulate briefly: these data with irradiated animals emphasize the quantitative relationship that exists between the lethal number of *Pseudomonas* organisms for the mouse, a dose of irradiation given the animal, the time postirradiation of bacterial challenge and, finally, the condition of the cells that phagocytize the microorganisms.

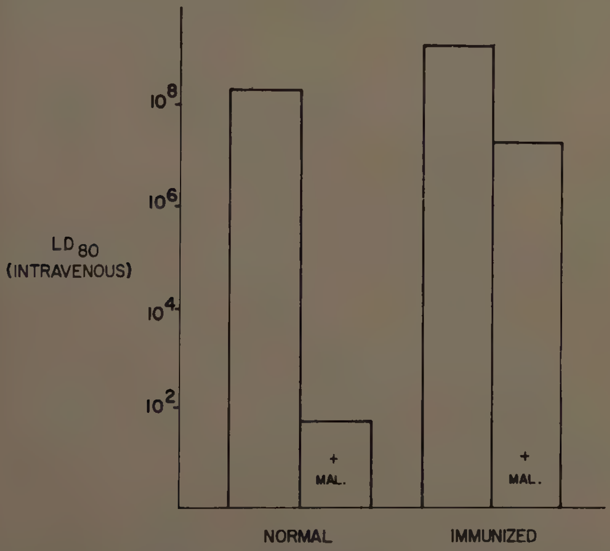

FIGURE 8. The lethal number of *Pseudomonas aeruginosa* for mice following treatment with malonate.

Malonate Toxicity

Following the work of Berry with tricarboxylic acid cycle inhibitors,[25,26] we investigated the effect of malonate on the resistance of mice to *Pseudomonas*.[27] FIGURE 8 illustrates the relationship we observed. In normal mice the LD_{80} was, as before, approximately 4×10^8. In mice given a series of injections of malonate on the day previous to challenge, we found that the resistance to *Pseudomonas* infection was completely eliminated; an inoculum of even 100 organisms was sufficient to cause death. However, if mice had been immunized previously with *Pseudomonas* antigen, their resistance to challenge following injections of malonate was considerably higher than in nonimmunized animals. There was some reduction in lethal dose from 10^9

to 10^7. Nevertheless, the difference between this group and the normal animals so treated was rather striking. Essentially these data illustrate the same kind of relationship that had been observed with irradiated animals. As we explored this relationship further, it became evident that in reality challenge with *Pseudomonas* was only an added insult-to-injury by malonate. In other words, the 10 to 100 organisms listed as necessary for death did not seem sufficiently great to explain the results. Therefore, in a subsequent study we investigated the resistance to malonate of normal and immunized mice. In this case *Pseudomonas* was not involved except where employed as immunizing antigen. Immunized mice received one subcutaneous injection followed by four intraperitoneal injections and were used in the experiments 7 to 10 days following the last injection.

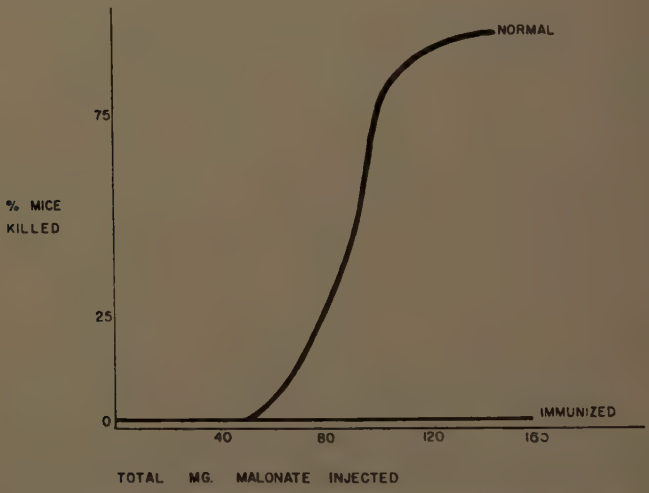

FIGURE 9. The toxicity of malonate (20 mg./mouse/hour) for normal and *Pseudomonas*-immunized mice.

The results of this experiment are presented in FIGURE 9. Once again the immunized mice were completely tolerant, whereas 80 to 100 mg. malonate (1 mg./gm./hr.) was sufficient to cause death of nonimmunized mice. What then was the relationship between immunization with *Pseudomonas* and resistance to malonate toxicity? In an extension of these studies immunizing agents other than *Pseudomonas* were used.

FIGURE 10 indicates what effect different immunizing materials and variations in the number and route of injections had on sensitivity of mice to malonate. It is quite apparent that three subcutaneous injections of *Pseudomonas* antigen did not have anywhere near the same protective effect as did a single intraperitoneal injection. At the same time it was possible to confer some degree of protection with multiple intraperitoneal injections of saline. The mechanism of action of the saline is unknown. Simple irritation of the peritoneal cavity may result in mobilization of cells that may be beneficial. However, the fact that some ten days elapsed after the last injection raises some doubt to this interpretation.

A recent report by Fruhman[28,29] has shown that saline, as prepared in the laboratory, may contain pyrogenic material and can cause both a febrile and a cellular response. If this is so, conceivably this could account for our results. Certainly the number of injections needed were considerable. The point of major interest is, of course, that previous treatment with even saline was adequate to cause a change in these animals such that they tolerated a drug to which they had not previously been exposed. In order to see whether this type of protection with malonate was also measurable at the cellular level, we repeated experiments similar to those we had done with irradiated animals, using the peritoneal exudate cells from malonate-treated mice given an inoculation of *P. aeruginosa*. The cells were harvested and maintained as before, and the number of viable organisms determined over a four-hour period.

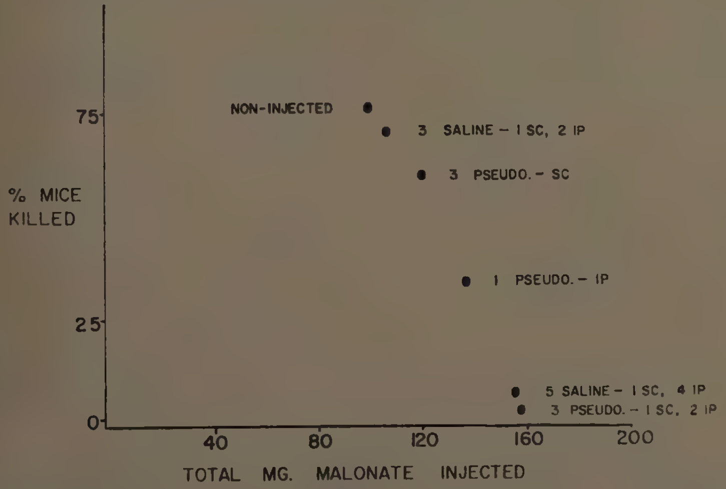

FIGURE 10. Protective effect of previous injections on malonate toxicity in mice.

The results of this experiment are presented in FIGURE 11. The cells from both normal and immunized animals adequately handle the *Pseudomonas* (that is, the number decreased rather than increased during the four-hour period). In cells from nonimmunized animals given malonate, essentially 40 per cent of the initial inoculum was viable at four hours; for purposes of comparison this was related to what was previously found with animals given 500 rad. X radiation and in which 100 per cent of the organisms survived four hours. Incidentally, cells obtained from saline-treated mice behaved toward *Pseudomonas* like those from nonimmunized animals although such mice were more resistant to malonate. Although saline injections protected against malonate toxicity, they did not protect against *Pseudomonas*. These results suggest a degree of specificity in protective activity referable to the immunizing agent.

In cells obtained from *Pseudomonas*-immunized animals, there was no effect on bactericidal activity following malonate treatment. Here also the results were similar to those obtained with immunized mice given 500 rad. X radiation. Protection through immunization did obtain at the cellular level as judged by these data, and it is also apparent that irradiation was somewhat more effective as an inhibitor of the normal cellular processes having to do with destruction of *Pseudomonas* than was malonate.

Metabolic Studies on the Interaction of Pseudomonas with Mammalian Cells

The studies with irradiation and malonate suggested that the interaction of these agents with cells occurred at some metabolic site related to that involved in the destruction of intracellular bacteria. We attempted to pursue this hypothesis using classical metabolic methods and devised experiments similar to those conducted by Stähelin *et al.*,[30] and others,[31,32] in which the metabolism of the phagocytic cell was studied in the Warburg.[33,34] Whereas other workers concentrated primarily on cells from rabbits and guinea pigs, we have for the most part limited our studies to mouse cells. We have employed peritoneal monocytes, HeLa cells, homogenates of liver and spleen, and isolated mitochondrial fractions of these tissues. We hoped in these studies to show some effect on the metabolism of the monocyte by *Pseudomonas* or, conversely, some effect of the monocyte on *Pseudomonas* metabolism.

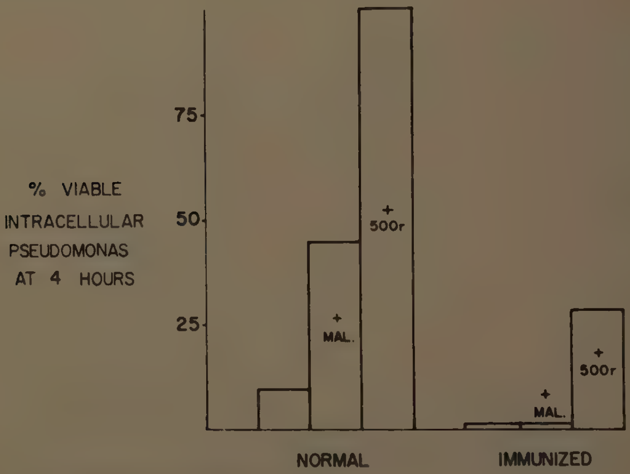

FIGURE 11. The survival of *Pseudomonas* in monocytes from normal and immunized mice.

A superficial review of our previous studies suggested that there was a basic metabolic difference between cells from immunized and normal animals. Perhaps if this were true such a difference might be amenable to detection. So far, however, we have found no qualitative difference in the metabolic activity of cells from normal or immunized animals. Also, in no case have we been able to detect any effect of phagocytic cells on bacterial metabolism. In these studies it was possible to use marker substrates for *Pseudomonas* metabolism. For instance, *Pseudomonas* attacks capric acid, whereas in no case do the monocytes utilize this substance. Therefore, any activity with this substrate when both bacteria and monocyte are present can be related to the microorganism. Similar marker substrates have not been found as yet for the monocyte. Apparently substances utilized by monocytes can also be attacked by *Pseudomonas*.

We have surveyed both the oxidative and glycolytic cycles of mouse cells and have found that, in general, oxidative metabolism is low but nevertheless measurable. Previous workers have concentrated primarily on the glycolytic aspects of phagocyte metabolism. These studies have generally indicated an increased glycolytic activity following phagocytosis.[31] Some reports[30] suggest that there is also a brief stimulation of oxidative activity during the process of phagocytosis. I wish to emphasize here that in our studies phagocytosis occurred *in vivo* prior to examination of the cells in the Warburg and that whereas many other studies have been concerned with cells removed several days after stimulation of the peritoneal cavity, in our work we have concentrated on those cells obtained a few hours following stimulation. These two differences I think may be important in the interpretation of the results obtained.

TABLE 2
OXYGEN UPTAKE BY INFECTED AND NONINFECTED MONOCYTES

Substrate	μl. O_2 uptake in 3 hours	
	Noninfected	Infected*
-(Endogenous)	53	33
Succinate	161	100

* 10^7–10^8 living *P. aeruginosa* were injected intraperitoneally into mice 2 hours prior to cell harvest.

TABLE 2 indicates the effect of infection on the oxygen uptake of peritoneal monocytes. Both the endogenous activity and the succinoxidase activity of the monocytes were substantially inhibited in the presence of *Pseudomonas*.

TABLE 3 shows the effect of *Pseudomonas* on a variety of cell preparations. It may be seen from these data that the organisms actively metabolize cellular materials. In the case of the spleen homogenate some inhibition of endogenous activity occurred following treatment with heat-killed *Pseudomonas*.

In TABLE 4 it may be seen that dead *Pseudomonas* also inhibit oxygen uptake of cells from both normal and immunized mice. The endogenous respiration of normal cells was inhibited 24 per cent and the inhibition of the succinoxidase system was 47 per cent. The cytochrome oxidase system in monocytes apparently resists inhibition by *P. aeruginosa*. With cells from immunized animals inhibition also occurred. However, the extent of inhibition was considerably less than in cells from nonimmunized animals. This is, we feel, a significant difference and an important point. That is, a population of cells from immunized animals, while still showing the same general pattern of metabolism and inhibition, was nevertheless quantitatively more resistant to *Pseudomonas* than similar populations from nonimmunized animals. Additional studies of this sort have shown that not only is the whole *Pseudomonas* organism inhibitory to various cells but also certain fractions of the organism may be equally inhibitory. These fractions were prepared by sonication and high speed centrifugation. The inert fractions, whether obtained from living or dead organisms, were equally toxic to the mammalian cells. An extension of these studies revealed that a chemically defined nonprotein fraction of *Pseudomonas* (obtained commercially as Piromen)* can produce all of the effects seen with intact organisms. Microgram quantities of Piromen quantitatively inhibit cell respiration (TABLE 5). The succinate activity of monocytes was inhibited by as little as 5 μg. of Piromen whether or not the Piro-

TABLE 3

THE EFFECT OF *PSEUDOMONAS* ON THE METABOLISM OF MAMMALIAN CELLS

Cell	μl. O_2 uptake in 3 hours		
	Endogenous	Live P.	Heated P.
—		28	
Monocytes*	26	308	27
Heated monocytes*		219	
Spleen homogenate†	52	940	34
HeLa + serum‡	132	1008	

* Approximately 10^8 cells/cup.
† 2 ml. of homogenate of 30 spleens in 30 ml. buffer.
‡ 10^6 cells + 1 ml. horse serum.

TABLE 4

EFFECT OF DEAD *P. AERUGINOSA* ON OXYGEN UPTAKE BY MONOCYTES FROM NORMAL AND IMMUNIZED MICE

Contents	μl. O_2 3 hours	
	N	I
-Endogenous	25	54
Succinate	105	104
PPDA	172	134
	Per cent inhibition	
-Endogenous + *P. aeruginosa*	24	13
Succinate + *P. aeruginosa*	47	30
PPDA + *P. aeruginosa*	0	0

---

* Travenol Laboratories, Morton Grove, Ill.

men was heated, indicating that the inhibitory activity was heat stable. It may be seen from this table also that, whereas the cytochrome oxidase system in monocytes was resistant to the action of the *Pseudomonas*, this system in liver mitochondria was sensitive to inhibition by Piromen. There seem to be metabolic differences in cells and cell particulates obtained from different areas of an animal. These studies will be reported in more detail elsewhere.

A summary of the effects of *Pseudomonas* on the metabolism of mammalian cells indicates that the organism, living or dead, and its component parts, including a chemically defined nucleic acid-polysaccharide fraction, all have rather decided toxic effects on the respiration of mammalian cells. In addition, it has been shown that the organisms actively attack and metabolize mammalian tissues. We infer from this that the over-all effect of *Pseudomonas* on mammalian tissue is one of direct toxicity. Growth of the organism is important only in so far as it contributes to the total amount of toxic substance available. Cells destroyed by the toxin may serve as substrate for the growth of more organisms that in turn increase the amount of available toxin. Such a pattern of alternation between toxic destruction of cells and bacterial multiplication could explain rather well the events leading to development of a *Pseudomonas* ulcer. Such a lesion would progress outward as more toxin was produced and more cells were killed. Healing and regeneration would be inhibited because new cells entering or developing in the area would also be subject to the accumulated toxins. Treatment with antibiotics might kill the organism but would not remove the toxin, therefore the lesion would persist. This hypothetical sequence of events fits very well descriptions of *Pseudomonas* lesions.[35,36]

TABLE 5

THE EFFECT OF PIROMEN ON OXIDASE ACTIVITIES OF MOUSE CELL PREPARATIONS

Preparation	Substrate	μl. O_2
Monocytes	Succinate	177
	Succinate + Piromen 5 μg./ml.	21
	Succinate + Piromen 5 μg./ml.	46
Mitochondria	PPDA	346
	PPDA + Piromen 3 μg./ml.	262
	PPDA + Piromen 3 μg./ml.	238

Discussion

Taken individually the data presented would seem somewhat irrelevant and difficult to relate to a concept of cellular immunity. Taken as a whole, however, we feel these data lend themselves very well to the development of such a concept. There is a certain consistency in the findings reported here and in the literature that binds them into a definite pattern. Irradiation, malonate toxicity, the toxic action of *Pseudomonas* and, indeed, the toxic action of various drugs and endotoxins[37-41] generally have one thing in common. Treatment with these agents results in destruction of cells. This is particularly noticeable in the generalized leukopenia that follows exposure to toxic substances. In those cases with drugs and endotoxins, where it has been studied, leukocytosis follows leukopenia and tissue regeneration follows tissue destruction. It is our contention that leukocytosis can occur only through proliferation of those cells (probably stem cells) that have survived exposure to the toxic agent and that, during the period of leukopenia and exposure, those cells most sensitive to the particular agent are the ones destroyed. In short, then, a selective process is initiated that results in the establishment of resistant cell lines within the individual.

Since we found that dead organisms were equally as toxic as living ones in the case of *Pseudomonas*, it seemed to us that what really happened during "immunization" was that those cells most sensitive to the organism were destroyed. The amounts of toxic bacteria used as immunizing antigen were not sufficient to kill the animals but only a percentage of their cells. Simply stated, our hypothesis is that, during infection, "immunization," or pretreatment, sensitive cells are eliminated; the cells that survive and repopulate an animal are naturally more resistant and are therefore selected. On subsequent exposure to infection an animal shows greater resistance because fewer of his cells are sensitive to the agent involved or, stated differently, more toxin is required to kill cells of the type that survived previous exposure. Incidentally, the animal shows greater resistance to other agents such as radiation and malonate that may act on similar metabolic loci.

A selective hypothesis of cellular immunity requires heterogeneity among cells of similar function and purpose. From a cytologic standpoint alone there seems to be little question that no two mammalian cells are identical. The literature is rich in reports of variations (in size, shape, cellular components, and biochemical activity) among similar cells differing in age, source, and habitat. In the studies of Thorbecke *et al.*[42] it was shown that the number of phosphatase rich cells in the liver changed following injection of endotoxin into animals. Other data[43] comparing the lysozyme activity of cells from the lungs with that of cells from peritoneal exudates indicate a decided difference in the amount of this enzyme present in similar cells found in different parts of the body. Such data support the idea of a heterogeneous cell population whose components are characterized by varying degrees of potential biochemical activity. Those cells having a particular biochemical potential might best resist a particular toxic agent. The concept of drug tolerance as established by the pharmacologist[3] falls into this same general category, and any data or discussion on this point may be interpreted in a similar way. Hook and Wagner[44,45] have shown enhanced resistance to peritoneal but not cerebral infection in mice following peritoneal exposure to endotoxin. Resistance to cerebral infection did, however, follow intracerebral inoculations with endotoxin. Such local immunity could be explained in terms of local selection of cell populations. Berthrong and Hamilton[46] have shown *in vitro* a selection of monocytes resistant to toxic effects of tubercle bacilli, and Holland and Pickett[14] had evidence of a similar phenomenon occurring with *Brucella*. A selection of drug-resistant mouse fibroblasts in tissue culture has also been reported.[47] Lieberman and Ove[48,49] established that mammalian cells in tissue culture may be selected for resistance to puromycin and 8-azaquanine. They emphasized that the parent (wild type) was heterogeneous and that mutations to drug resistance occurred at a fairly high frequency (4×10^{-6} per cell per generation). As continued selection occurred with higher and higher drug levels the mutation frequency increased (1×10^{-4}). These data are entirely consistent with our proposal of a selective mechanism of "cellular immunity."

Although the provocative report of Fong *et al.*[50] showing degrees of cross-cellular immunity between BCG-immunized and *Salmonella*-infected animals has been difficult to reconcile according to previous concepts, these data are readily understood when viewed in terms of a selective hypothesis. In terms of our concept of cellular immunity, such cross protection is rather to be expected in that a given agent would most assuredly select those cells that were most resistant to it. However, in so doing, it would also tend to select cells that for various reasons would be to some degree resistant to other toxic agents (TABLE 6). Elberg[12] has also noted that cells obtained from immunized animals survive for longer periods *in vitro*. Therefore, a sort of pseudospecificity would be established, a heirarchy perhaps of sensitivity to various agents. Such an interpretation could be placed on our finding of increased resistance to *Pseudomonas* only in irradiated and malonate-treated animals that were previously "immunized." In a sense, this type of cellular immunity is nonspecific in that there may be no direct relationship between the cell and the agent to which it is resistant. Resistance may only be fortuitous. Conversely it would be expected that an agent used in the pretreatment or "immunization" period would necessarily elicit some degree of specific resistance to subsequent challenge with that particular agent. With time, however, this resistance might gradually disappear as turnover of cells continued. Perhaps also there is a tendency to return to the *status quo* that originally was characterized by a more heterogeneous population of cells. Such turnover could account for the gradual loss in resistance that has been observed.

TABLE 6
SURVIVAL OF PERITONEAL EXUDATES *IN VITRO**

Source of cells	Per cent cells with nuclei stained by trypan blue	
	0 time	4 hours
Normal mice	4	20
Immunized* mice	0.5	2

* As previously described.[24]

Summary

We propose that the enhanced resistance or cellular immunity, characteristically observed in cells from animals treated with toxic agents, is not referable to an alteration of individual cells by the particular agent involved but rather to a selection of naturally resistant cells. We have presented evidence that treatment of mice with *Pseudomonas* antigen results in the destruction of those cells that are sensitive to this organism and its toxic

fractions. The partial destruction of a cell population would favor selective establishment of resistant cell lines that exhibit those attributes characteristic of cellular immunity.

Acknowledgments

Certain of the studies to which I have referred were carried out in the laboratory of C. Phillip Miller at the University of Chicago, Chicago, Ill.; I am grateful to him for his generous assistance and support of my efforts while in his laboratory. Without the work of my colleague R. S. Berk certain of these studies would never have been done. I am also grateful to Joan Becker and John Meacham for their excellent technical assistance.

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Discussion of Paper

Stuart Mudd (*Veterans Administration Hospital, Philadelphia, Pa.*): I have listened with very great interest to Nelson's exposition of fine experimental data. However, I do feel some anxiety about the elaboration of the interpretation that, as I understand it, supposes that clones of resistant organisms are selected from pre-existing resistant individual cells. Would it not be wise to consider this as a very tentative hypothesis pending thorough experimental test?

Nelson: I did not wish to imply that what I have proposed was any more than an hypothesis. The data presented here and much that has been written supports such a concept, but certainly much definitive work remains.

SERUM BACTERICIDAL ACTIONS

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Serum or plasma is the first body defense met by a parasite once it breaks through the skin or epithelial lining of viscera serving as a portal of entry. The bactericidal action of normal serum has been long recognized, therefore, as a factor in the total complex of "nonspecific" resistance to infection. The factors involved in the bactericidal activities of serum are highly complex and may vary widely among different mammalian species.

Serum bactericidal activities have been usually divided into two distinct groups. The components of the first group, which have been called β-lysins, are heat stable (60° C. for 30 to 40 min.), and therefore independent of complement (C′). These heat-stable substances are bactericidal against Gram-positive organisms such as *Bacillus anthracis*, *B. subtilis*, *Diplococcus pneumoniae*, and *Streptococcus pyogenes*. Several investigators (Tillett, 1954; Myrvik and Weiser, 1955) have reported a nonspecific increase in the bactericidal power of serum against these organisms during the acute phase of various illnesses with a subsequent return to normal levels upon recovery. These substances are not increased by immunization. Absorption of serum with one Gram-positive organism nullifies its killing power against other Gram-positive bacteria, but leaves unaltered its effect on Gram-negative bacteria. (Mackie and Finkelstein, 1932). Little is known of the mode of action or significance of these thermostable bactericidal substances. Myrvik and Leake (1960) have shown the necessity for two nondialysable components, in addition to the potentiation by bicarbonate ion, for the action of serum against *B. subtilis*. Although it is probable that these substances are concerned in host resistance, they are not a decisive factor in certain instances. Thus, in the case of anthrax, the serum of the naturally resistant dog has little effect against the anthrax bacillus, but serum of the susceptible rabbit is bactericidal (Nungester, 1951). An interesting finding of Hirsch (1960), moreover, indicates that these heat-stable bactericidal substances, or at least certain of the required components, are not really serum or humoral substances but are derived from blood platelets and formed during clotting. The bactericidal activity of these substances may well be absent, therefore, in circulating plasma.

In contrast to these heat-stable, platelet-derived substances, the activity of the second group of bactericidal substances is relatively heat-labile primarily because it is C′-dependent. Serum bactericidal action of this group is exerted on Gram-negative organisms and depends upon at least two substances, antibody (Ab) and C′. Other organisms including trypanosomes, leptospira, and treponema are also destroyed by the Ab-C′ system, but Gram-positive bacteria are generally insusceptible.

The standard method for determining the number of bacteria surviving serum action has been the plate count, but a simpler and more precise photometric assay similar to those used in antibiotic titrations has been described

(Treffers, 1956). In the photometric assay, the surviving bacteria are subcultured in broth. The broth terminates the bactericidal reaction and the surviving bacteria multiply. While still in the logarithmic phase of growth, the optical density attained by each culture is proportional to the number of bacteria surviving the original serum action. Assays of surviving bacteria have also been determined by conversion of tetrazolium salt, 2-, 3-, 5-triphenyl tetrazolium chloride to its reduced form, which gives a red color (Nagington, 1956). These methods do not distinguish between bactericidal and bacteriolytic action. For the estimation of immune bacteriolysis, Amano *et al.* (1958) have developed a method based on the photometric determination of released nucleic acid.

Titrations of serum for bactericidal activity against the Gram-negative bacteria have been confused often by a failure to recognize the dual role of Ab and C′. The bactericidal antibody titer of a serum cannot be estimated merely by testing varying amounts of a test serum. For example, without the addition of foreign or exogenous C′, an immune serum may give a lower titer than the preimmunization specimen (TABLE 1). This result arises because of the prozone phenomenon with larger amounts of immune serum and from insufficient C′ with smaller test quantities. With excess of added C′ (serum absorbed with heat-killed organisms at 0° C. to remove normal antibody), bactericidal antibody titrations may be performed with remarkable precision (FIGURE 1). Here the percentages of organisms killed are plotted against the logarithm of the amount of test serum on Curve A. This sigmoid curve may be rectified by conversion of the percentages of killed organisms to probits as seen in Curve B. The titer of the serum may be considered as the reciprocal of the amount of serum causing 50 per cent killing. Thus the bactericidal reaction constitutes one of the most precise and also sensitive methods for the measurement of antibody. For example, only 0.001 μg. rabbit antibody nitrogen are required for an endpoint against *Salmonella typhosa* 0901 (Treffers, 1958).

TABLE 1
BACTERICIDAL TITERS OF NORMAL AND IMMUNE RABBIT SERA AGAINST *SHIGELLA DYSENTERIAE*

	Without added C′	With added C′ (abs. g. p. serum)
Preimmunization serum	>136	227
Immune serum	25	19,000

Bactericidal C′ may be similarly titrated by adding an excess of antiserum. C′ is present in all mammalian sera and, within a particular species, its normal variation, at least as measured by the standard hemolytic system, is not very marked (Hartmann, 1958). The so-called normal or natural bactericidal antibodies against many of the Gram-negative organisms are also invariably present in sera. Although we are concerned with normal serum rather than with serum from animals that have been immunized, a "normal" animal or human may have had immunological experiences beyond the control of the investigator. These antibodies are often present even in the sera of germfree animals, but these animals are not entirely free of antigenic stimuli. In several well controlled experiments, however, it has been shown that certain so-called normal bacterial antibodies, absent in germfree animals, will be produced by these animals as a result of the introduction of living or dead bacteria in their diet (Springer, 1959; Wagner, 1959). At any event, whatever their origin these natural antibodies are invariably present in normal animals; they possess marked specificity of action (Mackie and Finkelstein, 1932; Adler, 1953; Muschel *et al.*, 1958) and act similarly to immune antibodies in the bactericidal reaction.

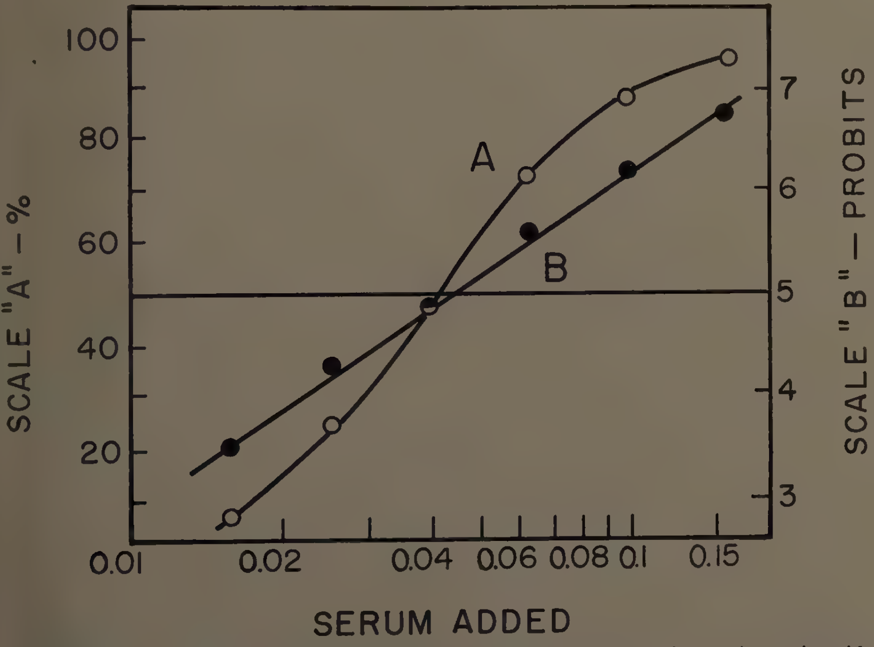

Figure 1. Comparative representations of inhibitory actions. Curve A = sigmoidal curve on linear scale of percentage inhibition. Curve B = linear representation resulting from same data plotted on probit scale. Abscissa scale logarithmic in each instance.

Not all investigators have agreed that the bactericidal antibodies in normal serum active against Gram-negative organisms are specific; some have considered them nonspecific or of limited specificity (Gordon and Carter, 1932). In the assumed absence of antibodies, serum bactericidal action against certain Gram-negative bacteria has been attributed recently to the properdin system (Wardlaw and Pillemer, 1956). Similar to Ab properdin required C′, but in contrast to Ab properdin was thought to be a single molecular substance lacking in serological specificity. When normal serum is absorbed, however, with the cells of a "properdin-sensitive" organism, activity against that organism is lost despite the presence of the components of the properdin system, while activity remains almost undiminished against serologically unrelated organisms (Osawa and Muschel, 1960). Thus, a substance uniquely removed by the test organism and that may be regarded as normal antibody is a *sine qua non* for serum action against Gram-negative organisms. In the presence of antibody, properdin solutions may enhance bactericidal activity, but since these solutions also contain lysozyme, amylase, alkaline phosphatase, other enzymes, and antibody to zymosan (Lepow *et al.*, 1959), this activating effect may be unrelated to properdin itself. Moreover, test systems that contained normal or immune Ab, C′, and Mg^{++} are highly bactericidal in the absence of detectable properdin. Insufficient evidence exists, therefore, to ascribe bactericidal action to the properdin system.

The mechanisms involved in the bactericidal reaction are not well defined although clues are provided by certain experimental findings. The requirements for Mg^{++} and Ca^{++} are similar to the cation requirements for immune hemolysis (Treffers, 1958), which have been interpreted as analogous to a cofactor requirement for an enzymatic process. Furthermore, calculations of the percentage of bacterial cell surface covered by Ab available for the bactericidal reaction has indicated that less than 1 per cent of the cell surface need be covered by antibody (Muschel and Treffers, 1956).

Bactericidal action may result, moreover, from the combination of Ab and C′ with surface antigens that need not be integral components of the cell. Adler (1952) showed that *Salmonella* organisms coated with extracts of *Escherichia coli* were killed by *E. coli* antiserums plus C′. It is unlikely, therefore, that C′ acts directly on the cellular structure. It seems that the reaction that occurs between C′ and an antigen-antibody complex in the immediate vicinity of the cell mediates the mechanism responsible for the destructive effect.

Several studies have indicated that the cell walls of susceptible bacteria constitute the locus for bactericidal action. The toxic O antigens of the bacteria, for example, have been shown to inhibit specifically the bactericidal power of both normal and immune serum (Cundiff and Morgan, 1941). As little as 0.10 mg. of protein-free toxic *S. typhosa* antigen sufficed to inhibit the activity of the homologous antiserum against *S. typhosa*, whereas 3.0 mg. of the Friedlander bacillus antigen was ineffective. These observations indicated that the endotoxins, located in the cell wall, constitute the major target antigens for both normal and immune serum in the systems studied. Although only a small percentage of the surface area of an organism need be covered by antibody for bactericidal action, the formation of bacterial protoplasts, or lysis, by the Ab-C′ system in conjunction with lysozyme (Muschel *et al.*, 1959) has also indicated that the cell wall is involved and attacked in serum bactericidal action. Apparently the Ab-C′ system may expose the mucopolysaccharide lysozyme substrate on the walls of Gram-negative bacteria or, possibly, the action of the Ab-C′ system may destroy or inactivate an inhibitor of lysozyme activity.

Linked possibly to the cell wall attack by serum is the fact that only organisms in a proper physiological state are susceptible to the Ab-C′ system. An extreme example of this principle is the fact that viable cells in a week-old culture have been found to be insusceptible to killing by serum (Muschel and Treffers, 1956). Recent work by Michael and Braun (1959) has demonstrated that those amino acids known to stimulate the metabolic activity of bacteria enhanced serum bactericidal action, while other amino acids protected against this action. These authors postulated that the effect of serum on the cell wall may not suffice to cause bacterial lysis. The lysis may occur only if wall-damaged bacteria "grow out of their damaged walls." In these respects, incidentally, serum bactericidal action mimics the bactericidal action of penicillin.

The Gram-negative bacteria that are susceptible to serum bactericidal action include species of *Vibrio*, *Salmonella*, *Proteus*, *Shigella*, *Hemophilus*, and *Brucella*. Rough strains are generally more sensitive than smooth strains. Certain organisms such as the mouse and chimpanzee virulent Ty_2 strain of *S. typhosa* are relatively resistant to normal serum, but may be easily killed by the combined action of highly diluted antiserum and C′. Among strains of *S. typhosa* and *E. coli*, a significant association has been noted between the O-inagglutinability of the strains and their resistance to normal sera (Felix and Pitt, 1951; Muschel, 1960). Moreover, resistance to serum may be an important determinant of mouse virulence with Gram-negative organisms as tested by intraperitoneal challenge (Rowley, 1954) or in rabbits by intravenous challenge (Rowntree and Pappas, 1960). In humans, strains resistant to serum are more apt to be isolated from blood, whereas sensitive strains are particularly prone to occur in urine or stool (Rowntree and Rantz, 1960).

As might be expected, the defense apparently afforded by the serum bactericidal reaction may be diminished by substances inimical to the reaction. Recent studies by Beeson and Rowley (1959) have suggested that the kidney's peculiar vulnerability to coliform bacterial infections may be related to that organ's anticomplementary action. This anticomplementary action has been attributed to the glutaminase activity of kidney tissue, which liberates ammonia and inactivates the fourth component of C′.

Not all Gram-negative bacteria are killed by serum. Some strains of *Paracolobactrum ballerup*, *S. typhimurium*, and *S. paratyphosa C* are completely insusceptible to both normal and immune serum under the usual test conditions. The factors responsible for this resistance are not readily apparent and they are probably multiple. For example, although O-inagglutinability is generally correlated with serum resistance within a particular species, one of the refractory organisms, *S. paratyphosa C*, is O-agglutinable. Pronounced anticomplementary action of the resistant organisms, an obvious possible explanation for this resistance, has proved not to be involved (Osawa, 1960).

The discovery by Nicolle *et al.* (1953) that the Vi content or O-inagglutinability of *S. typhosa* was decreased by growth of that organism at temperatures above or below 37° C. presented a possibility for serum bactericidal action against resistant Gram-negative organisms. It was found that serum bactericidal action against susceptible organisms was enhanced by cultivation of the organisms at temperatures above 37° C. and that *P. ballerup*, ordinarily an insusceptible organism, cultivated at elevated temperatures became markedly susceptible not only to immune but also to normal sera (TABLE 2). *S. paratyphosa C*, an O-agglutinable strain, remained insusceptible, however, when cultivated at elevated temperatures (Osawa, 1960). In these experiments, the organisms were cultivated for 16 hours at different temperatures on meat-extract agar and then an appropriately prepared inoculum was allowed to react with serum for 1 hour. Variations in the 1 hour-reaction temperature from 37° C. to 43° C. did not appreciably affect the test results. The loss of resistance by cultivation at elevated temperatures did not result in a genetic change in the organisms since reincubation of serum-sensitive *P. ballerup* at 37° C. for 16 hours resulted in almost complete restoration of serum resistance. Susceptibility to serum bactericidal action acquired by growth at elevated temperatures is not a simple phenomenon, moreover, and does not occur when *P. ballerup* is grown on brain-heart infusion agar instead of meat-extract agar. These results suggested that the pyrogenic response of the host to endotoxins of the Gram-negative bacteria may lead to enhanced Ab defenses, at least in some cases, mediated by the bactericidal reaction.

Differences among bacteria are equaled by differences among mammalian species in their serum bactericidal action. Normal sera of the human, guinea pig, bovine, and rabbit are effective bactericidal agents. Serum of the mouse is lacking in bactericidal action (Marcus *et al.*, 1954), and that of the hamster has been found to be lacking (Fujii and Nozawa, 1955) or extremely weak in our own laboratory. In the case of the mouse, the lack of activity has been found to result from a relative deficiency of the second and third C′ components. Normal antibody of the mouse in conjunction with C′ of other species, however, is capable of bactericidal action (Muschel and Muto, 1956). With the exception of the mouse, there is little or no correlation between the hemolytic and bactericidal activity of C′. Cow complement, which is inactive in the standard hemolytic system, for example, is a very potent bactericidal C′ in many systems.

Table 2

Bactericidal Titers of Normal and Immune Rabbit Sera Against *P. ballerup* and *S. paratyphosa* C Cultured at Various Temperatures

Rabbit serum	37° C.	39° C.	41° C.	42° C.	43° C.
	Bactericidal titer versus *P. ballerup*				
Preimmunization	<5	<5	286	400	264
Antiserum versus *P. ballerup*	<5	<5	16,700	21,300	12,500
	Bactericidal titer versus *S. paratyphosa* C				
Preimmunization	<5	<5	<5	<5	<5
Antiserum versus *S. paratyphosa* C	<5	<5	<5	<5	<5

Finally, the extent to which the bactericidal reaction is involved in protecting the host against disease is not clear. In certain instances, high levels of bactericidal antibody have not protected humans against microbial invasion and clinical disease. On the other hand, significant associations between the virulence of microorganisms in animals and their resistance to serum bactericidal action have been observed. In addition, the presence of serum bactericidal antibodies in successful active and passive immunization experiments cannot be easily ignored. The Ab-C′ bactericidal system undoubtedly operates *in vivo* and thus aids the host in defending itself against invading Gram-negative microbes. Its role may depend on the individual balances between infection and resistance. The question remains whether a moderate reduction in the number of organisms, with help from other immunity factors such as phagocytes, will be decisive for recovery or whether all, or nearly all, of the organisms must be killed by the bactericidal reaction before resistance becomes effective.

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INACTIVATION OF ENDOTOXIN BY HUMORAL SYSTEMS*

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Endotoxin is the only component common to the Enterobacteriaceae that appears capable of contributing significantly to the toxic state characteristic of infection with Gram-negative bacteria. Despite constant exposure to these noxious agents, mammals are singularly free from their toxic effects. However, little is known about mechanisms by which the host metabolizes these lipopolysaccharide complexes.

Studies in this laboratory have shown that the blood of mammalian species contains at least two systems that can inactivate endotoxin *in vitro*. One of these, clearly distinguishable from properdin, complement, and specific antibody, has been designated endotoxin detoxifying component (EDC). For measurement of the inactivation of endotoxin by EDC, mice bearing 6-day-old implants of Sarcoma 37 were employed. The standard dose of 10 μg. of *Serratia marcescens* endotoxin regularly produced marked hemorrhagic necrosis in the tumors. This tumor-damaging action of the endotoxin was abolished by prior incubation of endotoxin with the specimen under test.[1] Serum and citrated plasma from nine mammalian species were examined for EDC activity. The comparison showed consistently that the serums of most species displayed little or no activity, whereas the citrated plasma of every species was highly potent;[2] only in the case of a few species such as man, chimpanzee, and rat was serum active to an appreciable degree. The nature of the striking difference in the EDC potency of serum and citrated plasma was investigated. It was shown that EDC activity was inhibited by divalent cations, notably Ca, Mg, Mn, and Ba. The high level of EDC activity provided by citrated plasma appeared to be achieved by virtue of the calcium-binding properties of the anticoagulant. Similar potency was obtained with the use of other calcium-binding anticoagulants, such as oxalate, fluoride, EDTA, or by the collection of blood through a cation-exchange resin. In contrast, heparin, an anticoagulant that does not complex calcium, was without effect. Furthermore, the addition of these calcium-binding agents to serum rendered it as potent as plasma in the inactivation of endotoxin.

Dialysis against distilled water markedly reduced the potency of ion exchange plasma; activity could be fully restored by recombining the dialysate and the dialyzed plasma. This property of the dialysate withstood incineration. The ashed dialysate could be replaced by physiologic levels of phosphate plus bicarbonate. Restoration of activity was also effected by supplementation of the dialysate with calcium-binding anticoagulants. These findings indicated that the suppression of EDC activity by calcium was not direct, but was probably mediated through its effects on an anionic component of plasma that is also required for inactivation of endotoxin by EDC.[3]

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* In the oral presentation of recent work in this laboratory, literature citations were not discussed. However, they are to be found in the bibliographies of the papers cited at the end of this article.

The conditions routinely employed for interacting endotoxin and plasma were one hour at 37° C. at 7.6, the normal *p*H of plasma. No inactivation was discernible at 30° C. or lower temperatures; the reaction was inhibited significantly at *p*H 5, proceeded well at the normal *p*H of serum or plasma, but was optimal at *p*H 9. The rate of the reaction was determined primarily by *p*H and concentration of the reactants. It was shown that 10 to 20 minutes sufficed to destroy the tumor-damaging potency of the endotoxin. Endotoxins derived by different isolation procedures from various colonially smooth and rough Gram-negative species were all inactivated by human plasma, but the quantity of this reagent required for inactivation varied widely for the different endotoxin preparations.

The alterations in endotoxin effected by EDC were also examined in other host reactions. It could be shown that the interaction with a system possessing these distinctive characteristics reduced or abolished the capacity of endotoxin to produce fatal shock in thorotrast-prepared rabbits, to prepare for the local Shwartzman reaction, to potentiate the dermal necrotic action of epinephrine, and to elicit pyrogenic and leucopenic effects (W. R. Keene and M. Landy, unpublished observations).

Inasmuch as endotoxins also possess individually characteristic antigenic properties, the effect of interaction with EDC on the immunologic activity of typhoid endotoxin was also studied.[4] A single intravenous injection of 10 μg. of this endotoxin suffices to evoke good levels of typhoid O agglutinins in rabbits. After appropriate interaction *in vitro* with plasma or serum of mice, of rabbits or of human beings, this quantity of endotoxin no longer elicited significant levels of typhoid O antibody. The conditions under which antigenicity for rabbits was eliminated were the same as those previously shown to be required for abolition of tumor-damaging and other effects of endotoxin. Furthermore, the same interaction also altered its capacity to precipitate specific antibody as measured in quantitative precipitin tests. The course of the precipitin reaction of the altered endotoxin was strikingly similar to that of the haptenic polysaccharide derived from the endotoxin by hydrolysis with mineral acid. It was considered significant that the typhoid endotoxin, although rendered nonantigenic for the rabbit, still displayed *in vitro* the immunologic specificity of the polysaccharide moiety. Consequently, the changes produced by EDC did not affect the polysaccharide configuration that confers on somatic antigens their immunologic specificities. The loss of antigenicity appeared to reflect mainly a loss of macromolecular attributes. It was visualized that the endotoxin macromolecule had been split into much smaller polysaccharide units that no longer had an affinity for phagocytic and antibody-forming cells. It would, therefore, be disposed of by the host so rapidly as to preclude its functioning as an antigen.

The studies were extended to ascertain whether endotoxin, in its native state on the bacterial surface, was also susceptible to inactivation by an agent with the distinctive features of EDC.[5] Two characteristic attributes of the endotoxin of intact *Salmonella typhosa*, the induction of tumor damage and the stimulation of antibody formation, were the test situations employed to measure alterations in the endotoxin by this humoral system. Incubation of citrated human plasma with bacilli, killed by heat or chemical treatment, eliminated antigenic and tumor-damaging activity of the organisms. The potency of matched specimens of serum and plasma from individual donors was compared. Serum and heparinized plasma had but little effect on the bacterial endotoxin, whereas plasma collected in citrate or through a cation-exchange resin and serum with added Ca binding agents were all highly effective in eliminating toxic and antigenic activities. These experiments showed that divalent cation suppressed the effect of the system in blood. Furthermore, conditions of time and temperature for this inactivation were similar to those previously established for alteration of isolated endotoxin by EDC.

The toxic and antigenic activities of viable bacilli, on the other hand, were unaffected by EDC. These activities were eliminated by fresh serum, but the requirements for this effect were quite different from those that had been established for inactivation of endotoxin of heat-killed bacilli. Serums of all animal species tested, with the exception of the mouse, inactivated tumor-damaging and antigenic properties of *S. typhosa.* This inactivating capacity was found to parallel their bactericidal activity against the typhoid bacilli. The components in fresh, normal serum required for bactericidal action were found to be specific antibody, the recognized components of complement and divalent cation. Serum rendered deficient in any of the constituents of this system no longer was capable of eliminating toxic or antigenic potency of the bacilli nor of exerting a bactericidal effect. Furthermore, the quantity of serum required and the kinetics of the reaction *in vitro* were essentially the same for producing both kinds of effects. Although killing of the typhoid bacilli by serum was invariably accompanied by loss of their toxic and antigenic effects, it remains to be determined whether these changes in the endotoxin complex of the bacteria are critical for the bactericidal action of fresh serum. There can be little doubt, however, that at some stage of the complex interaction between serum and these Gram-negative bacteria, endotoxin is the substrate for one or more enzymatic reactions. This destruction of the endotoxic capabilities of viable organisms has important implications for the host-parasite relationship.

Recently we have reported[6] the presence of an endotoxin-inactivating agent in animal tissues. Rabbit liver was thoroughly perfused with chilled saline to remove blood. Slices, homogenates, or cell fractions of this organ were then interacted *in vitro* with *Serr. marcescens* endotoxin, and the effects of these tissue preparations on the tumor-damaging and the pyrogenic potency of the endotoxin were determined. Liver slices had no discernible effect, but the homogenates inactivated the endotoxin.

After removal of nuclei and mitochondria, the homogenate, still containing the microsomal component, altered the endotoxin. However, neither the microsomal component alone nor the supernatant freed of microsomes alone was effective. Recombination of these two fractions, however, restored the capacity to inactivate endotoxin. This activity of the liver homogenate was exerted over a wide *p*H range, but its effectiveness was greatly enhanced at *p*H 9 to 9.5. It withstood dialysis, was unaffected by the addition of Ca or Ca-binding agents, and was destroyed by exposure to 52° C. for 15 minutes.

Inasmuch as reticulendothelial cells are known to clear endotoxin from the circulation and have been implicated in its ultimate disposal, it was at first thought that this inactivating potency might be associated with tissues rich in these cells. However, similar detoxifying activity has also been found in organs such as kidney. It is therefore unlikely that this agent is restricted solely to reticuloendothelial cells. How extensively it is distributed in host tissues remains to be determined.

As these studies progressed it has become increasingly apparent that these host systems possess enzymatic attributes and that their interactions with endotoxins are complex. It is now known that many factors determine the outcome of these reactions:

The endotoxin (*substrate*) *employed.* Many different endotoxins have been examined and, although it was possible to inactivate all of them, important differences were observed in the concentration of plasma required for different products. It is not known whether these differences are associated with particular microbial source, method of isolation, or composition, that is, proportions of polysaccharide, lipid, and protein.

The host product (*enzyme*). Its effectiveness and potency differ, depending on whether it is derived from fresh serum, plasma, or tissues, the animal species from which it is obtained, and the method of its collection (anticoagulant) or preparation (extraction procedure).

The conditions for interaction in vitro. The temperature, time, *p*H and, especially, the relative concentration of host product and endotoxin, play decisive roles in the outcome of the reaction.

The indicator system employed to disclose the results of the in vitro interaction. It has been ascertained that the alterations effected in the endotoxin involve its capacity to evoke each of the varied host reactions thus far examined. These endotoxic effects were, in every instance, either greatly diminished or abolished as a consequence of interaction with these enzyme systems. However, as regards their suitability for the assay of endotoxin and its inactivation, the various reactions of experimental animals to endotoxin differ greatly. The advantages and limitations of these various host responses have previously been considered. The procedures with which we have had the most extensive experience, and which were found to be useful, were hemorrhagic necrosis in Sarcoma 37 implants in mice, antibody production in rabbits and, most recently, the pyrogenic response of rabbits (unpublished observations). Even these three indicator systems differ considerably as regards minimal dose of endotoxin effective in evoking the reaction, uniformity of response, and dose effect (magnitude of response in relation to the dose).

Our findings have made it evident that in blood and tissues of animals there are present systems, with enzymatic characteristics, that are abundantly capable of degrading endotoxins *in vitro* so as to render them nonreactive for the host. These systems differ from one another as regards the experimental conditions under which they are operative. Their relationship is not known, but the possibility is recognized that they may be more closely associated than present information indicates. For example, the system in cells could be visualized as being a precursor of EDC in blood. Furthermore the effects of the antibody-complement system on the endotoxin of viable organisms may represent a "preparatory" effect on the bacteria for subsequent action by EDC that is normally present in the same serum specimens. Whether these activities involve three distinct systems or will be found to reflect a single kind of enzymatic effect, it is clear that the host has the capability of coping with endotoxin either on Gram-negative bacteria or in a "soluble" state.

It was pointed out that, under appropriate conditions of test, inactivation of substantial quantities of endotoxin was accomplished by rather small increments of serum, plasma, or tissue homogenate. On the basis of such criteria the endotoxin-inactivating potential of the host would have to be considered as indeed great. The fact that mammals remain free of the harmful effects of endotoxin, despite their constant exposure to these noxious agents, further attests to the host's possession of efficient means of metabolizing these polysaccharide complexes. Although supporting evidence is still to be obtained,[7] it seems reasonable to assume that these humoral and cellular systems contribute to the defense of the host and may thus constitute an important factor in natural resistance.

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SOME METABOLIC ASPECTS OF HOST-PARASITE INTERACTIONS IN THE MOUSE TYPHOID MODEL*

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The influence of host metabolism on susceptibility to bacterial infections, primarily but not exclusively infections with *Salmonella typhimurium*, has been studied for several years. It was first shown that several of the well known inhibitors of specific steps in the tricarboxylic acid cycle, when injected at approximately the same time as the pathogen, reduced dramatically the mean survival time of experimental animals.[1-4] Also effective in reducing survival time were several intermediates of the same metabolic cycle.[3] The extent to which the animals, albino mice, were succumbing to the infection itself, to the toxicity of inhibitor or intermediate, or to a combination of the two was impossible to assess. Until there is some basis for attributing cause of death to specific biochemical lesions in infections with organisms other than exotoxin producers, assessment will continue to be difficult if not impossible. It was for this reason that the total number of viable pathogens in the mouse was determined.[5,6] One of the advantages of using infections with *S. typhimurium* is the highly selective culture media available for its enumeration. These studies revealed a remarkably constant number of culturable cells of *S. typhimurium* at the moment of death irrespective of survival time and experimental procedure used to alter it. Approximately 10^9 cells were consistently recoverable from the animals at death. To this extent, it seemed justifiable to assume that altered host metabolism, under the conditions of the experiments, reduced survival time by permitting the "lethal" population of viable pathogens to be reached more rapidly.[7]

Within the framework of contemporary knowledge, further pursuit of this type of experimentation seemed to offer promising returns only if developments in related studies would be awaited. As Dubos,[8,9] Pappenheimer,[10] and others[11-13] have suggested, the disease state in the infected animal may be the result of competition between host and bacterial parasite for an essential metabolite such as a growth factor, a vitamin, or an amino acid, or it may be the consequence of antimetabolite formation that interferes with an essential step in host cell metabolism by one mechanism or another. With the mouse and *S. typhimurium*, comparatively crude yet potentially revealing techniques yielded only negative evidence for metabolic competition between host and parasite (unpublished observations). It seemed essential, therefore, to paraphrase a statement once made by Racker,[14] "to search for a thin metabolic trail left by the pathogen in the forest of metabolism." This search led us to a closer look at some of the effects of bacterial endotoxins in the hope that, at least in mouse typhoid infections, it might be possible to relate disease symptoms to their presence, despite the important experiment of Hill *et al.*[15]

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approximately two decades ago. These workers, it might be recalled, obtained an inbred strain of mice highly resistant to an endotoxin derived from *S. typhimurium.* The animals were normally susceptible, however, to infection with the same organism. The experiment does not necessarily prove the absence of a role of endotoxin in the pathogenesis of the disease since the locus of action of injected endotoxin might well be different from that of endotoxin produced in the course of an infection.

Methods

Mice were infected intraperitoneally with 10^5 cells of *S. typhimurium*, strain SR-11, contained in 0.5 ml. of a saline dilution of a 17-hour brain-heart infusion broth culture. Viable pathogen counts were made on infected mice at different times postinfection by a technique previously described.[5] In brief, this consists of homogenizing in a Waring blendor the carcass of the whole mouse after it has been skinned and its feet and tail removed. Appropriate dilutions, cultured on SS agar, are incubated and typical colonies enumerated.

Liver and muscle glycogen were determined by the method of Kemp and Kits van Heijningen.[16] Urine nitrogen excretion was evaluated by micro-Kjeldahl analysis on overnight samples collected in the manner described by Berry and Smythe.[17] Blood nonprotein nitrogen (NPN) was measured by micro-Kjeldahl on trichloroacetic acid filtrates of samples pooled from two or three mice, the number depending upon the severity of illness and the volume of blood shed following decapitation with sharp scissors.

Washed cells of *S. typhimurium* were suspended in saline, enumerated by dilution count, and killed by pasteurization at 60° C. for 30 min. Appropriate dilutions of this vaccine containing the desired number of organisms in 0.5 ml. were injected intraperitoneally as a source of bacterial endotoxin.

Results

Glycogen changes in liver and muscle of infected mice. FIGURE 1 presents graphically the changes in liver and muscle glycogen observed in mice at different times postinfection. During the first 12 hours liver glycogen is reduced to less than 1 per cent (5.5 per cent is normal) and muscle glycogen drops to about two thirds of normal. During the second and third day of the disease there is an increase in carbohydrate reserve that then declines to near depletion levels at the time of death. As some indication of the state of the infection during the changes, the viable count of *S. typhimurium* in the whole mouse is plotted against days postinfection.

During the first day the bacteria increase 100-fold. For the succeeding four days a tenfold multiplication occurs, and during the final day there is another log unit rise to 10^9 cells, the mean number at death. The changes in viable cells reveal nothing about the number of dead pathogens, that is, the amount of endotoxin that might be produced during the course of the disease. The remainder of this report is devoted to the results of experiments designed to throw some light on this question.

It should be emphasized that data derived from infected mice typically show increasing variability with time postinfection. Under the conditions of these experiments, the first animals die at about 72 hours and from 90 to 95 per cent are dead at six days. Obviously, those animals near death should show changes of a more uniform nature, regardless of elapsed time postinfection than animals studied at a specific time. Nevertheless, the behavior of the "average" animal will be described throughout this paper.

Voluntary food consumption of infected mice. If infected mice fail to eat during the first day postinfection, the drop in liver and muscle glycogen just described would be attributable to inanition. This possibility was tested by placing animals in individual cages and offering them a sucrose and tryptone solution as the sole source of nutriment for an overnight period of about 17 hours. The results are presented in TABLE 1. Only during the first period did the majority of mice fail to consume most of the fluid. Thereafter about 80 per cent of the total volume made available to the mice was drunk. From the third night, there were always a few animals drinking none of the solution and these were dead when found or died shortly thereafter. It may be that for some hours prior to death animals refuse food, but this is not clearly established by the data. Thus a simple explanation, if not necessarily the correct one, is available to explain the initial decrease and subsequent rise in glycogen reserves in infected mice. The terminal decline in carbohydrate seen in FIGURE 1 cannot be attributed directly, however, to inanition.

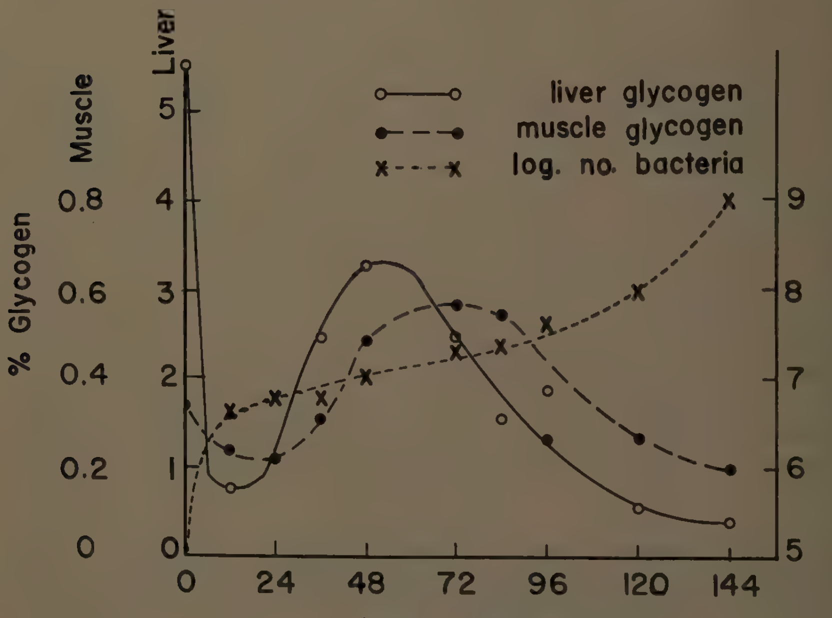

FIGURE 1.

Also presented in TABLE 1 are the percentages of total nutritive solution drunk by mice injected with known numbers of heat-killed cells. As few as 2.5×10^7 cells give a clear reduction in volume of fluid consumed (70 per cent), while ten times as many cells stop drinking completely. It is of particular interest to raise the question of a possible linkage between quantity of endotoxin present in the infected mice and the suppression of nutritive fluid intake. Note that mice injected with 1×10^8 heat-killed cells drank the same percentage of fluid as mice did during the first overnight period postinfection (28 per cent). The viable pathogen count at this time (24 hours) is about 5×10^6 (FIGURE 1) and hence too small a number of cells to produce the effect as equivalent heat-killed organisms. Moreover, the presence of larger numbers of living salmonellae on subsequent days postinfection when the fluid intake was 80 per cent of the control value makes it questionable as to the extent to which living cells can be assumed to be involved in depressing food consumption.

These findings suggest an explanation, however, for the carbohydrate changes plotted in FIGURE 1. The initial drop would appear to be due, at least in part, to the failure of animals to eat. The rise in glycogen seen during the second day postinfection would follow the increase in food intake, but the terminal decline occurs during a period of continued eating. Since it is known that bacterial endotoxins deplete carbohydrate reserves and, indeed, inhibit glycogenesis[18] then it is merely necessary to assume an increasing accumulation of endotoxic substances during the later periods of the infection to account for the results in FIGURE 1.

TABLE 1

PERCENTAGE OF TOTAL VOLUME (5 ML.) OF A SUCROSE AND TRYPTONE SOLUTION CONSUMED DURING AN OVERNIGHT PERIOD OF ABOUT 17 HOURS BY INFECTED MICE AND MICE INJECTED WITH A KNOWN NUMBER OF HEAT-KILLED CELLS OF *SALMONELLA TYPHIMURIUM*

Hours postinfection	Per cent of nutritive solution consumed by		Number of heat-killed cells per mouse
	Infected mice (%)	Mice given heat-killed cells (%)	
6–23	28	99	Control
30–47	84	100	5×10^5
54–71	82	92	1×10^6
78–95	80	70	2.5×10^7
101–119	78	28	1×10^8
		0	2.5×10^8

Influence of heat-killed S. typhimurium on urinary nitrogen excreted in response to ACTH. In an earlier publication[17] the effect of injections of known numbers of heat-killed cells of *S. typhimurium* on the response of mice to ACTH was described. These data are presented graphically as the solid line in FIGURE 2 and in the appropriate columns of TABLE 2. Urinary nitrogen excretion more than doubles when 0.5 to 2 or more units of ACTH are injected into control animals.[17] There is a decrease in urinary nitrogen when a heat-killed vaccine is given at the same time as the ACTH. The linear nature of the decline in urinary nitrogen output under these conditions makes it possible to use the procedure as an assay for number of dead salmonellae or their endotoxic equivalent. There is convincing evidence, soon to be presented in a separate publication, to suggest that endotoxin alone produces this effect.

The physiological basis for the assay is not understood at the present time. Experiments aimed at elucidating it have eliminated some of the possible explanations, but much work remains before it is resolved. However, when the test was applied to infected mice, the results described in the next section were obtained.

Urinary nitrogen excretion in response to ACTH in infected mice. Urine collected overnight from a pair of infected mice given a subcutaneous injection of ACTH was analyzed for nitrogen. On the basis of the results, endotoxin or materials acting similar to it can be detected during the first night postinfection as indicated by the dashed curve in FIGURE 2 and by the data of TABLE 2. For the next two nights less endotoxin appears to be present within the animal but, again on the fifth night, the urinary nitrogen level was depressed. This suggests an initial destruction of bacteria in the early period postinfection and their subsequent detoxification during the second and third days of the disease. In this phase of the infection there are sufficient viable cells present (FIGURE 1) between 10^7 and 10^8, to be detectable by the assay, but the extent to which they contribute to the slight lowering of urinary nitrogen excretion in response to ACTH cannot be evaluated. The mice, during the fifth night postinfection, according to the results shown in TABLE 2 and FIGURE 2, excrete a smaller amount of urinary nitrogen than previously, and this may be interpreted as evidence for a second release or production of endotoxinlike material. All data point to the likelihood of a terminal phase of the disease in which increasing endotoxinlike effects are manifest, but animals have not been studied so far at the moment of death because of obvious difficulties.

TABLE 2

URINARY NITROGEN EXCRETION FOLLOWING INJECTION OF TWO UNITS OF ACTH DURING AN OVERNIGHT PERIOD OF ABOUT 17 HOURS BY INFECTED MICE AND MICE INJECTED WITH A KNOWN NUMBER OF HEAT-KILLED CELLS OF *SALMONELLA TYPHIMURIUM**

Hours postinfection	Mg. urinary nitrogen excreted per mouse per 17-hour period by		Number of heat-killed cells per mouse
	Infected mice	Mice given heat-killed cells	
6–23	17.5 ± 3.1 (15)	32.8 ± 6.9 (8)	0
30–47	24.8 ± 4.5 (15)	31.3 ± 5.1 (8)	5×10^6
54–71	28.0 ± 2.7 (14)	23.8 ± 3.4 (15)	5×10^7
102–119	16.8 ± 3.3 (14)	17.0 ± 3.0 (15)	2.5×10^8
		11.6 ± 2.0 (16)	5×10^8

* Each value is the mean ± the standard deviation for the number of determinations shown in parentheses.

Influence of heat-killed S. typhimurium on nonprotein nitrogen of blood. The relationships between blood NPN in mice at different times postinfection and 17 to 18 hours after the injection of a known number of heat-killed cells of *S. typhimurium* are presented graphically in FIGURE 3 from the data of TABLE 3. Note that the only significant change in blood NPN of infected mice was found after five days (119 hours). On the other hand, the injection of 2.5×10^8 heat-killed cells is without statistically significant effect,

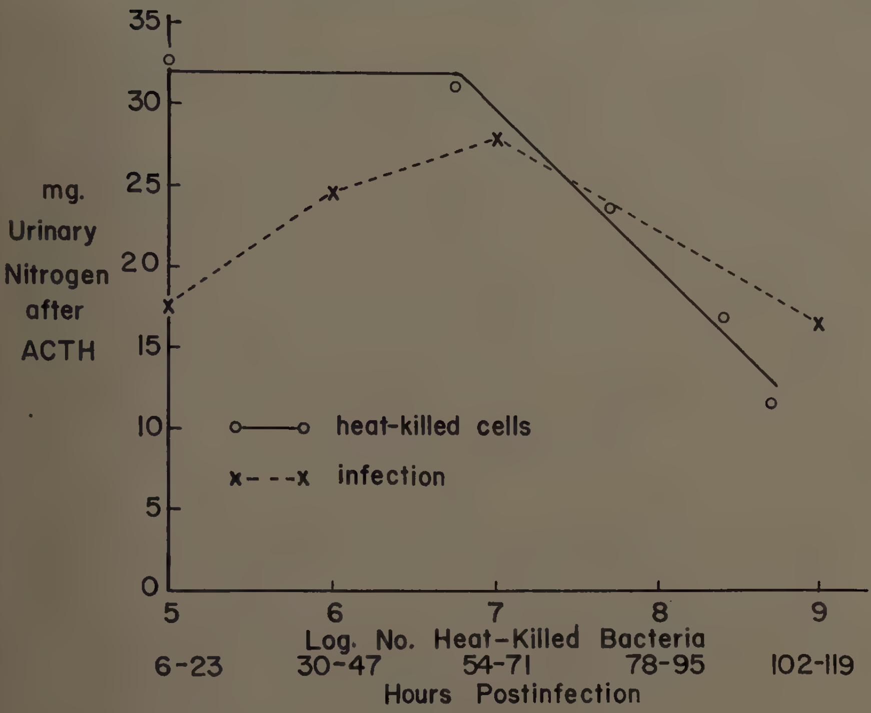

Figure 2.

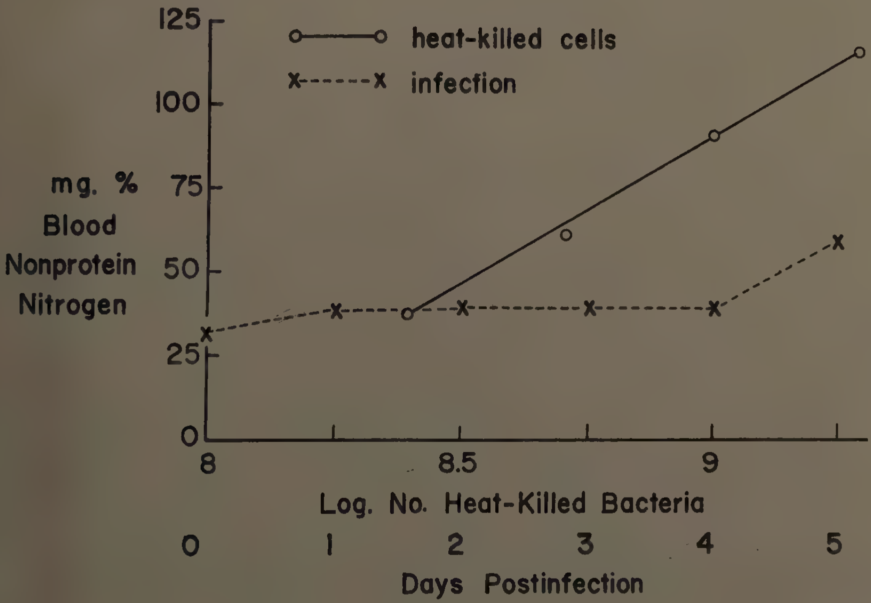

Figure 3.

while 5 × 10^8 bacteria produce approximately a 70 per cent increase in NPN. An injection of 10^9 organisms elevates NPN nearly threefold, while 2.0 × 10^9 cells nearly quadruples it. The NPN under these conditions is directly proportional, therefore, to the logarithm of the number of heat-killed cells injected, as demonstrated by the linearity of the graph. If it is assumed that the endotoxin released within the infected mouse is the cause for the increase in blood NPN on the fifth day, then the equivalent of nearly 5 × 10^8 heat-killed cells are present at this time. It is important to point out that 5 × 10^9 heat-killed salmonellae are 100 per cent lethal to mice within a period of 48 hours. Thus the 5 × 10^8 cells estimated by blood NPN assay fall below a lethal dose. Individual animals from which the average number 5 × 10^8 was derived had NPN values, however, as high as those found in mice injected with 10^9 cells. Some of the animals, therefore, might possibly contain at the time of death, according to this reasoning, close to a lethal amount of endotoxin.

TABLE 3

NONPROTEIN NITROGEN IN BLOOD OF INFECTED MICE AND OF MICE INJECTED WITH KNOWN NUMBERS OF HEAT-KILLED CELLS OF *SALMONELLA TYPHIMURIUM**

Hours postinfection	Mg. per cent nonprotein nitrogen in blood of		Number of heat-killed cells per mouse
	Infected mice	Mice given heat-killed cells	
24	33.2 ± 4.2 (7)	33.1 ± 3.4 (8)	0
48	37.0 ± 4.3 (8)	37.4 ± 3.1 (7)	2.5 × 10^8
72	38.1 ± 4.6 (6)	59.5 ± 14.1 (15)	5 × 10^8
96	37.6 ± 6.4 (8)	90.4 ± 18.6 (6)	1 × 10^9
120	58.9 ± 16.8 (11)	115.8 ± 15.7 (6)	2 × 10^9

* Each value is the mean ± the standard deviation of the number of separate determinations shown in parentheses.

Discussion

Three different criteria have been used in this work in an attempt to estimate the amount of endotoxin in mice infected with *S. typhimurium*. These are: (1) the quantity of nutritive solution consumed during an overnight period; (2) the amount of urinary nitrogen excreted during a 17-hour period following a subcutaneous injection of ACTH; and (3) the nonprotein nitrogen content of blood. Each of these, when evaluated with heat-killed cells of the same pathogenic organism, can be employed as an assay for endotoxin. Used in combination, one serves as a check against the others. Thus, the infected mouse during the first 24 hours contains endotoxin equivalent to from 1.0 to 1.8 × 10^8 heat-killed cells, as seen in the data of TABLE 4. The blood NPN values are not definitive at this time but do permit an estimate of less than 2.5 × 10^8 cells. Between 54 and 71 hours postinfection, on the basis of fluid intake, 10^7 cells are present, while the urinary nitrogen assay gives a value of 1.6 × 10^7 cells (TABLE 4). Blood NPN at this interval is not at all informative. In the third interval, the period 102 to 119 hours postinfection, 2 × 10^8 heat-killed cells or their equivalent are present, according to urinary nitrogen determination, while 5 × 10^8 cells are indicated by blood NPN assay. At this time, by food intake measurement, a number completely out of line, 1 × 10^7 cells, is found. It must be admitted that the numerical agreement found by different tests may be fortuitous or it may, hopefully, have significance.

None of the "assays" for endotoxin are based, so far as we know, on physiological changes uniquely attributable to the poison. Changes in blood NPN and in appetite, for example, can be produced by substances other than endotoxin. Similarly, the urinary nitrogen excretion in response to ACTH could conceivably be depressed in various ways. Concerted efforts to find them, on the other hand, have failed. The results described, therefore, must be considered strictly as tentative evidence for endotoxin release and only in this particular infection. As previously mentioned, it should be borne in mind that the effect of endotoxin formed *in vivo* in infected animals might act in a manner quite different from heat-killed cells injected intraperitoneally or, for that matter, by any other route. The approach described does offer, however, some promise of revealing the basis of certain metabolic disturbances in mouse typhoid.

TABLE 4

SUMMARY OF "ASSAYS" FOR ENDOTOXIN CONTENT EXPRESSED AS "EQUIVALENT HEAT-KILLED CELLS" IN MICE AT DIFFERENT TIMES POSTINFECTION WITH *SALMONELLA TYPHIMURIUM*

Assay	"Equivalent number of heat-killed cells" at times specified postinfection		
	6–23 hr.	54–71 hr.	102–119 hr.
Per cent of nutritive fluid consumed	1 × 10^8	1 × 10^7	1 × 10^7
Urinary nitrogen excreted after ACTH	1.8 × 10^8	1.6 × 10^7	2 × 10^8
Nonprotein nitrogen content	<2.5 × 10^8	<2.5 × 10^8	5 × 10^8

Summary

Liver and muscle glycogen in mice infected with *S. typhimurium* at a dosage level that kills the first animals at 72 hours and 90 to 95 per cent at 6 days drop during the first 12 to 24 hours. There is a steady rise toward normal for liver and above normal for muscle glycogen during the next 2 days, but thereafter both reserves decline to near depletion levels at death. The viable count of pathogens throughout this time steadily increases but most rapidly during the first and last day.

In an attempt to relate the carbohydrate changes to the release of endotoxin in the infected mice, a series of measurements on food consumption, urinary nitrogen excretion following ACTH injection, and blood nonprotein nitrogen were carried out. In each instance determinations were made at different times postinfection and compared to values found when known numbers of heat-killed cells of *S. typhimurium* had previously been injected.

On the basis of these data, there appears to be a release of readily detectable amounts of endotoxin during the first day of infection. For the next two

to three days only small amounts of endotoxin seem to be present, but just before death large quantities appear.

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Part III. Some Rickettsial and Viral Problems

SOME ASPECTS OF VARIATION IN RICKETTSIAL VIRULENCE

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Rickettsiae are among the most notorious pathogens of man and it is well to include them in a discussion of microbial pathogenicity. Information on biological and some of the biochemical aspects of their pathogenicity is not lacking. Difficulty is encountered when this diverse and fragmentary information is analyzed and an attempt is made to find unifying principles. Cohn (1960) has recently presented an integrated picture of the biological activities and metabolic properties of rickettsiae. I propose to cover some of the same ground, utilizing the information on rickettsial variation, and to present some pertinent data from our laboratory. I shall be guided by the premise that rickettsiae are relatively simple microorganisms and that the same basic reactions govern all their major activities.

Pathogenicity for Experimental and Natural Hosts

The discussion that follows refers primarily to *Rickettsia prowazekii*, but examples will be freely drawn from work carried out with the other species. The yolk sac of the chick embryo (Cox, 1938, 1941) has been the tissue of choice for the growth of large numbers of microorganisms and for the performance of a great variety of experiments. The susceptibility of the chick embryo, although somewhat lower for recent isolates, is identical to that of the cotton rat (Fox, 1949) in the case of established strains. Fuller (1953) has shown, in a painstaking experiment, that the susceptibility of the louse infected by membrane feeding is exactly the same as that of the cotton rat injected intracardially. The actual number of rickettsiae contained in an infectious unit was found by Price *et al.* (1958) to be remarkably low. With most of his purified preparations 10 rickettsiae were sufficient to infect 50 per cent of the cotton rats. The dose required to infect man, the primary host of *R. prowazekii*, is not known, but is undoubtedly small. Precise information is available, however, on the susceptibility of man to two other rickettsiae, *R. tsutsugamushi* (Ley *et al.*, 1952) and *Coxiella burnetii* (Tigertt and Benenson, 1956). It was shown that, under conditions approximating those occurring in nature, man is as susceptible as the laboratory animal of choice in each case. Therefore, it is not unreasonable to add man to the list of hosts that are infected by the smallest infective unit of *R. prowazekii*.

Thus typhus rickettsiae find comparable cellular sites for penetration and growth in four unrelated hosts, namely, the chick embryo, cotton rat, louse, and possibly man. There are points of similarity in the four rickettsial-host cell interactions. For example, yolk sacs infected with *R. prowazekii* contain maximal titers just before the death of the embryo (Cox, 1948), while with *R. rickettsii* the peak is reached approximately 24 hours after death (Bell and Pickens, 1953). This difference can be attributed to the fact that *R. rickettsii* causes greater injury and kills its host during an earlier phase of its growth than *R. prowazekii*. The same type of difference is encountered in the louse: rectally infected lice, at the time of death, are not as heavily infected with *R. ricksettsii* as with *R. prowazekii* (Weyer, 1954).

The response of the host may vary considerably. Infection is invariably fatal for the louse. The chick embryo, inoculated on the 6th day of incubation, almost always dies before the time of hatching. On the other hand, in the cotton rat the response is so balanced that one egg infectious unit is sufficient to produce solid immunity, but as many as 10^5 units do not produce pronounced illness (Anderson, 1944). Because of this equilibrium, which favors the host, the cotton rat is not as well suited for primary isolation of rickettsiae as the guinea pig, which is slightly less susceptible (Fox, 1949) but not as effective in curbing the infection. With doses above $10^{5.5}$ infectious rickettsiae, the cotton rat too succumbs to the effects of infection and dies.

Paradoxically, pathogenesis of rickettsiae is studied quite well (1) in a host—the mouse—by a procedure not eliciting infection, and (2) in an *in vitro* test—hemolysin—that, superficially, does not resemble the *in vivo* reaction. There is good evidence, however, that these two interactions reproduce at least one important step that leads to infection, possibly cell penetration. Mouse toxin (Gildemeister and Haagen, 1940) and rabbit or sheep cell hemolysin (Clark and Fox, 1948) are intimately associated with the viability of the rickettsiae. Of the many attempts to separate the various properties of typhus rickettsiae, only moderate irradiation with ultraviolet light has been successful in selectively destroying infectivity (Allen *et al.*, 1954).

The action of the toxin has been described in detail by several investigators (Wattenberg *et al.*, 1955; Neva and Snyder, 1955). The toxin is usually demonstrated by injecting at least 10^6 microorganisms intravenously into mice. Depending on the concentration, the animals die in 1 to 6 hours. The toxin can be neutralized by immune serum before but not after injection, which suggests that the attachment of the rickettsiae to their site of action is very rapid. The effect is described as an increase in capillary permeability that is not accompanied by other manifestations of vascular damage. The other changes are secondary and consist of flow of plasma into the tissues and hemoconcentration. The blood pressure is maintained until the sudden fall that occurs just before death.

An immediate reaction cannot be produced in the susceptible cotton rat, even by very large doses of mouse toxin (Anderson, 1944). However, by 2 to 6 days after injection of a sufficient number of microorganisms, a syndrome is elicited that is entirely analogous to that produced by the toxin in the mouse. It is more difficult to evaluate pathogenesis in man, but there are many features of the illness (Snyder, 1959) that can be well interpreted to be the result of the action of the toxin.

The hemolytic action of rickettsiae, because of its simplicity, offers an excellent opportunity for the study of the biochemical aspects of pathogenicity, but its relationship to virulence needs to be defined. It is true that a fatal toxemia can be produced in the rabbit by the intravenous injection of large numbers of rickettsiae. Death can be directly attributed to the destruction of red blood cells and massive release of potassium (Paterson *et al.*, 1954). However, hemolysis is not a prominent feature of the syndrome in the animal susceptible to infection or in the mouse, and the cells of these hosts are not lysed by rickettsiae *in vitro*. However, it should also be kept in mind, as explained in a later section, that almost all the factors that have thus far been found to affect rickettsial toxin also affect hemolysin. Thus, it is a good working hypothesis that toxin and hemolysin are manifestations of the same basic action, the former affecting the permeability of the vascular endothelium, the latter affecting the permeability of certain red blood cells.

Rickettsial Metabolism

Rickettsiae have some of the basic characteristics of bacteria, namely, they divide by binary fission (Schaechter *et al.*, 1957*a*), have a cell wall envelope (Schaechter *et al.*, 1957*b*), and possess a moderately active energy-yielding metabolism. Glutamate is the chief substrate, as first shown by Bovarnick and Snyder (1949). Although rickettsiae are relatively impermeable to products of intermediary metabolism, it has been shown that utilization of glutamate proceeds through steps involved in the Krebs cycle (Wisseman *et al.*, 1952). A transaminase responsible for the production of aspartate as a by-product has also been demonstrated (Bovarnick and Miller, 1950; Hopps *et al.*, 1956). The terminal respiratory pathway was also partly elucidated and it was found that it is mediated through a flavin enzyme-iron-cytochrome system which probably includes cytochromes A_1 and B_1 (Hayes *et al.*, 1956). Rickettsiae are capable of converting oxidative energy into high energy phosphate bonds, with formation of adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and inorganic phosphate. More recently, Bovarnick *et al.* (1959) have demonstrated that small but highly significant amounts of methionine-S^{35} are incorporated into rickettsiae suspended in a cell-free medium containing known metabolites and a small amount of protein. This uptake most likely represents true protein synthesis.

Numerous examples can be cited that clearly indicate that virulence of rickettsiae is closely associated with metabolic activity. Glutamate enhances the hemolysis of sheep cells by rickettsiae (Snyder *et al.*, 1954) and their penetration into cells *in vitro* (Cohn *et al.*, 1959). Divalent ions that enhance glutamate oxidation and metabolic inhibitors that depress glutamate oxidation (Wisseman *et al.*, 1951) or phosphorylation (Bovarnick, 1956) have the same enhancing or depressing effect on hemolysis and cell penetration.

Physiologic Variation

Despite their limited metabolic activity and impermeability to some small compounds, typhus rickettsiae are greatly affected by the composition of suspending media, can lose or regain highly phosphorylated compounds, and, as a result, are subject to a wide variation in physiologic state. These changes affect to approximately the same degree infectivity for eggs, toxicity for mice, and hemolysin. Bovarnick *et al.* (1950, 1953) have shown that the stability of purified suspensions of rickettsiae is favored by a basal salt solution high in K^+, rather than Na^+, glutamate, diphosphorydine nucleotide (DPN), sucrose, and albumin. The requirement for K^+ may reflect an adaptation to an intracellular environment, while sucrose and albumin most likely prevent leakage of metabolites into the medium. The activity of rickettsiae is reduced to an appreciable extent when they are suspended in isotonic salt solution and are frozen and thawed or just maintained at 0° C. for 18 hours. This reduction is accompanied by loss of DPN; it can be prevented by adding DPN to the suspending medium or the lost activity can be restored by incubating the rickettsiae with DPN at 33° C. for 3 hours (Bovarnick and Allen, 1954, 1957*a*). Activity is also lost when rickettsiae are incubated for several hours at 36° C. in the absence of substrate. This loss is accompanied by a fall in ATP level and it can be prevented by incubating the rickettsiae with ATP or glutamate. The ATP content and biological activity of rickettsiae can be restored by incubation with glutamate (Bovarnick and Allen, 1957*b*). A similar loss and restoration involving nucleic acids has also been reported (Cohn *et al.*, 1958). It is obvious, from the data available thus far, that a typhus rickettsia requires an intact energy-producing apparatus in order to penetrate a cell and initiate infection or display its virulence.

R. rickettsii undergoes important physiologic changes that in many respects resemble those of typhus rickettsiae. However, there are sufficient differences to pose puzzling problems of interpretation. It was shown by Spencer and Parker (1930) that, if *Dermacentor andersoni* ticks infected with a virulent strain of *R. rickettsii* are refrigerated for several months, the rickettsiae are no longer highly virulent for the guinea pig, although they immunize it against challenge with a virulent strain. Virulence is reestablished when the ticks have a blood meal or are incubated at 37° C. for 48 hours. Price (1953*a*, 1954) has obtained convincing evidence that this phenomenon cannot be attributed to a difference in the number of rickettsiae in the tick, to a population change, or to spontaneous mutation. It is, then, a true physiologic change affecting the virulence of the rickettsiae for the guinea pig, other animals, and possibly man. However, rickettsiae in either physiologic state are virulent for the chick embryo and a single egg passage reestablishes virulence for the guinea pig. Gilford and Price (1955) reproduced the phenomenon *in vitro* by incubating yolk sac suspensions of spotted fever rickettsiae at 25° C. for 60 hours. Without substrate all activity was lost but, when *p*-aminobenzoic acid (PAB) was added, virulence for the chick embryo was retained but not for the guinea pig. With DPN, coenzyme A (CoA), or *p*-hydroxybenzoic acid, an important rickettsial metabolite (Snyder and Davis, 1951) virulence for both hosts was retained. Avirulent rickettsiae were converted to virulence by incubation with DPN, CoA, or normal tick extract. In all of these experiments a good correlation was obtained between guinea pig virulence and adsorption of rickettsiae onto guinea pig tunica cells *in vitro*.

When the physiologic changes of typhus and spotted fever rickettsiae are compared, similarities are apparent with the chief exception that the latter retain their virulence for the chick embryo. However, it is plausible that *R. rickettsii* is reactivated in the yolk sac of the chick embryo, in the same manner as in the previously described conditions, and that related biochemical systems are involved in the physiologic changes of the two species.

Rickettsial Genetics

Genetic variation among rickettsiae is considerably less spectacular than physiologic change. Possibly the only example of a reproducible genetic change is the phase variation of *Coxiella burnetii*, described by Stoker and Fiset (Stoker, 1953; Stoker and Fiset, 1956; Fiset, 1957). The significance of these findings for other rickettsiae is not known.

Strain differences are commonly encountered in nature in *R. rickettsii*. Price (1953*b*) characterized strains R, S, T, and U on the basis of decreasing virulence for the guinea pig and showed that these differences did not extend to other pathogenic properties. Virulence for the chick embryo, toxin and hemolysin yields from infected yolk sacs, and rates of respiration in the presence of glutamate or Krebs intermediates appear to be similar. Furthermore, there is no correlation between pathogenicity for the guinea pig and for man. The first three strains may undergo the previously described physiologic changes within limits of strain characteristics. Strain variation in *R. tsutsugamushi* appears to be somewhat more pronounced. Irons (1946) demonstrated that the virulence of scrub typhus strains for the mouse correlates very well with virulence for man. Strain variation among naturally occurring typhus rickettsiae appears to be slight.

In the laboratory genetic changes have been produced only with some difficulty. In fact, the stability of some strains has been remarkable. For example, the Breinl strain, isolated approximately in 1920 in Warsaw, Poland, and maintained by passage in guinea pigs for many years, and, more recently, in over 100 passages in eggs, proved to be virulent for man 37 years later (Fox *et al.*, 1957). Immediately following the establishment of the yolk sac method of inoculation, Cox (1941) passed the Iowa strain of *R. rickettsii* serially in eggs 240 times. The original isolate was not highly pathogenic for the guinea pig, but its virulence increased to the 15th passage, remained high until the 50th, then gradually decreased. By the 240th passage the strain produced only slight fever, lasting one or two days, in the guinea pig or rhesus monkey, but both animals were immunized by very small doses. Mouse toxin production was not affected (Bell and Pickens, 1953).

The Madrid E strain of *R. prowazekii* received much wider attention. This strain was isolated from a severe case of typhus in 1941 and its egg passage history is similar to that of the Iowa strain. The harvest from the 255th passage was characterized in detail by Perez Gallardo and Fox (1948). The chief change consisted in a marked reduction in virulence and ability to grow in the guinea pig and a slight reduction in virulence, but not infectivity, for the cotton rat. Pathogenicity for man appeared to have been lost entirely and complement-fixing antibodies were elicited with regularity only when the dose was in excess of $10^{5.5}$ egg ID_{50} (Everitt *et al.*, 1954; Fox *et al.*, 1954). Other properties, including virulence for the louse, were not changed. Strain E has been used for much of the previously described biochemical investigations without any indication that it differs from the other typhus strains.

It is apparent that the genetic changes involving virulence briefly reviewed above, in contrast to physiologic variation, involve a relatively minor modification of the affinity of the rickettsial cell for natural substrates and not an easily definable change in biochemical reactivity.

Drug Resistance

Genetic changes involving increased resistance to inhibitory substances have been studied in our laboratory (Weiss *et al.*, 1957, 1959*a* and *b*, 1960). They appear to be of somewhat different nature. TABLE 1 lists the drug-resistant substrains of *R. prowazekii*, Madrid E, that have been developed in our laboratory during the past several years. The substrains were obtained by passing the parent Madrid E strain serially in eggs in the presence of increasing amounts of drug. When drug resistance became apparent the substrains were isolated, in the absence of drug, by the limiting dilution technique that is a rough equivalent of pure culture isolation.

TABLE 1

DRUG-RESISTANT SUBSTRAINS OF *RICKETTSIA PROWAZEKII* (MADRID E) DEVELOPED IN OUR LABORATORY

Substrain	Probable No. of mutational steps	Drugs to which resistant	Origin	No. serial drug passages in eggs*
Parent	0	None	Madrid E†	0
P^{sr}	1	*p*-Aminobenzoic acid (PAB)	Parent	16
P^r	2	PAB	Parent	37
P^{rr}	3	PAB	P^r	21
A^r	4	PAB and acetylsalicylic acid	P^{rr}	9
Q^r	2	2,3-Dimethyl-quinoxaline-1,4-dioxide (quinoxaline)	Parent	20
E^r	1	Erythromycin	Parent	12
PQ^r	4 or 5	PAB and quinoxaline	P^{rr} and Q^r	2
PQ_1^r (4 isolates)	4 or 5	PAB and quinoxaline	P^{rr} and Q^r	1
EQ^r (3 isolates)	2	Erythromycin and quinoxaline	E^r	1

* Following the indicated numbers of drug passages each substrain was isolated from a limit-dilution passage (<25 per cent infection) without drug.

† The Madrid E strain had undergone approximately 280 passages in eggs since its isolation when received in our laboratory.

One of the purposes for the production of these strains was a search for evidence of genetic transfer among rickettsial strains. Two such experiments are indicated in TABLE 1. The P^{rr} and Q^r substrains, resistant to single drugs, were mixed and injected into eggs that had received both PAB and 2,3-dimethyl-quinoxaline-1,4-dioxide (quinoxaline) to which these strains were resistant. Isolates resistant to both drugs were obtained in two experiments. In the first experiment, a substrain (PQ^r) was isolated by limiting dilution from the second drug passage. In a more recent experiment several isolations (PQ_1^r) were made after a single drug passage. However, unequivocal evidence that the substrains resistant to two drugs were the product of genetic transfer was not obtained. In fact, in experiments involving the E^r, erythromycin-resistant, and Q^r substrains, EQ^r isolates were obtained from both the test group containing E^r and Q^r and from the control group containing only E^r. My associates and I have come to the conclusion, to our sorrow, that quinoxaline resistance can be acquired under certain conditions during a single drug passage.

However, an observation has emerged from these studies that bears on virulence. Properties unrelated to drug resistance were not found to have changed in most of these strains. However, quinoxaline resistance appeared to be associated with some reduction in virulence for both the chick embryo and the cotton rat. As shown in previous publications (1959*a*, 1960), with the parent, P^{rr}, A^r, and E^r substrains there is an inverse linear relationship between number of infectious units injected into the chick embryos and length of time of their survival after infection. Furthermore, embryos infected with these strains almost invariably die within the period of incubation of 13 days. With Q^r, on the other hand, there is a different relationship between dose and survival, embryos die about 1 day later than those receiving an equivalent number of parent strain rickettsiae and, at the end of the incubation period, several infected embryos survive. The change encountered in PQ^r is even more drastic, even though it is not apparent with high concentrations of rickettsiae. With lower concentrations a great number of infected embryos survive and, in contrast to the other strains, infection in some cases cannot be demonstrated by smear but only by subinoculation into eggs. Accompanying this change in virulence for the chick embryo there is a reduction in virulence and infectivity for the cotton rat. In the case of Q^r the change is slight, but with PQ^r cotton rats are not killed by the largest doses that can be injected and immunity is produced only by the inoculation of large doses. Mouse toxicity did not appear to have changed.

TABLE 2
VIRULENCE FOR THE CHICK EMBRYO OF *RICKETTSIA PROWAZEKII* (MADRID E) SUBSTRAINS AS INDICATED BY THE RATIO OF THE ID_{50} TO THE LD_{50}

Substrain	ID_{50}/LD_{50}	Substrain	ID_{50}/LD_{50}
Parent	1.2	Q^r	38
P^{rr}	1.2	PQ^r	250
A^r	2.2	PQ_1^r	38*
E^r	1.0	EQ^r	30†

* Geometric average of results with 4 isolates.
† Geometric average of results with 3 isolates.

The results obtained with PQ^r proved to be unusual. PQ_1^r isolates obtained in the second experiment resembled Q^r somewhat more than PQ^r. For example, in most cases 1 mouse LD_{50} corresponded to 0.1 cotton rat LD_{50}, a significant difference from both parent (0.6 LD_{50}) and PQ^r ($<$0.04 LD_{50}). The virulence for the chick embryo could best be expressed as the ratio between the arithmetic ID_{50} and LD_{50} in a given dose, a high ratio representing low virulence, and vice versa. TABLE 2 presents such ratios for the various isolates. The results clearly indicate that the quinoxaline-resistant substrains are appreciably less virulent than those that are susceptible.

As shown in TABLE 1, PQ^r and PQ_1^r were isolated from mixtures of P^{rr} and Q^r. If these strains were produced by a mechanism of genetic transfer, we must surmise that the quinoxaline and virulence markers were transferred at the same time. If, on the other hand, these isolates are P^{rr} substrains that acquired quinoxaline resistance in 1 or 2 drug passages, it would appear that quinoxaline resistance is often associated with loss of virulence. EQ^r was obtained from only one substrain, E^r. Therefore, in this instance only the latter event could have occurred. In any case, the association of quinoxaline resistance and loss of virulence for the chick embryo has appeared in at least two independent experiments and is likely more than a coincidence.

TABLE 3 presents an effort to determine whether these two properties can be separated. A PQ_1^r isolate was passed in eggs 10 times in the presence of both drugs in one series of experiments and 10 times in the absence of drugs in another. Both lines retained an appreciable level of resistance to quinoxaline, although the level of the drugless line may have been slightly reduced. The line passed with drugs remained relatively avirulent for the chick embryo, as indicated by a high ID_{50}/LD_{50} ratio. On the other hand, the line passed without drugs regained its virulence, as indicated by a low ID_{50}/LD_{50} ratio. These properties appeared to be stable and were not changed by a single additional passage of the opposite kind, drugless if the rickettsiae had been passed with drugs or vice versa. We can therefore conclude that, although the two characteristics, quinoxaline resistance and loss of virulence for the chick embryo, are often associated, they can be separated.

TABLE 3
VIRULENCE FOR THE CHICK EMBRYO OF PQ_1^r ISOLATE FOLLOWING PASSAGES IN EGGS

Passage history	ID_{50}/LD_{50}
PQ_1^r	15
PQ_1^r + 10 drug passages	35
PQ_1^r + 10 drug + 1 drugless passages	55
PQ_1^r + 10 drugless passages	2.1
PQ_1^r + 10 drugless + 1 drug passages	1.6

Conclusion

In assessing the over-all picture of variation in rickettsiae, two features stand out. First, physiologic fluctuations can be very great and express themselves in changes in a large number of biological properties. An example is the loss and reacquisition of hemolysin, mouse toxicity, and infectivity for the chick embryo of typhus rickettsiae. Second, genetic variation, as for example reduction in virulence or appearance of drug resistance, have been relatively specific. Mutations in physiologic activity that manifest themselves in a series of seemingly unrelated biologic changes had not previously been found. Our results with the Q^r substrains have not yet been exploited, but are possibly the first indication that it might be possible to obtain a genetic change that has a broader influence on the physiology of the rickettsia.

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CHEMISTRY OF VIRAL INFECTED CELLS*

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Upon the basis of limited data available ten years ago, a concept of viral infection became popular in which the cell was redirected after infection to the exclusive production of virus. These original data (Cohen, 1949) that concerned the quantitative accounting of the nucleoproteins during the infectious sequence, offered a direct line of investigation into the mechanism of this redirection and into the genesis of the cytopathic effect of the T_2 phage of *Escherichia coli.*

Since the early work of Cohen, there have been few attempts to establish an accounting of large molecules during infection with other viruses, particularly in well-defined systems of animal virsues. Such investigation is complicated by the fact that the yield of virus from infected animal cells is frequently low relative to the cell mass. What determines the yield is an interesting problem in itself, as well as how that viral production leads to cell destruction. At present there is no simple answer. It may be that the cytopathic effect terminates the viral production rather than the latter mediating the cytopathic effect.

However, a quantitative analysis of cells and fractions thereof infected with poliovirus was undertaken in our laboratory several years ago, and it was quickly established that, although the amount of virus formed was small, there were indeed massive increases in the total RNA and protein of the cell during the infectious process (Ackermann *et al.*, 1959). These results have been confirmed elsewhere by microspectrophotometric analysis of single infected cells (G. Barski, personal communication; Tenebaum, 1957). They are further supported by the series of studies of the proteins and enzymes of cells infected with poliovirus (Matzelt *et al.*, 1958). If a viral cytopathic effect requires a specific or locally restricted synthesis of an amount of material large relative to the cell mass, this is available in the poliovirus infection.

Since the report of these changes in cells induced by infection with the poliovirus, alterations of nucleic acid content of cells have been observed during the infectious sequence of several viral systems. Data concerning this point were collected in many laboratories and are not entirely comparable in all respects, but they have been summarized in TABLE 1. In these examples, the infected cells do not seem to be devoted to the exclusive production of materials that constitute what is usually regarded as the viral body.

Alterations of Nucleic Acid Content Induced by Virus

In each case where kinetic studies were undertaken to follow these changes, increases in nucleic acid were observed to occur progressively during the latent period. Usually the synthesis begins within an hour after the initiation of

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infection and continues until the appearance of new infectious activity. The pattern (FIGURE 1) for vaccinia virus (Loh *et al.*, 1959) conforms approximately with that described for infections with poliovirus (Ackermann *et al.* 1959), herpes virus (Newton and Stoker, 1958), pseudorabies (Kaplan and Ben-Porat, 1959), adenovirus (Ginsberg and Dixon, 1959*a*), and T_2 bacteriophage (Cohen, 1949).

A quantitative accounting of the various species of nucleic acids found in cells after infection with any particular virus is difficult to make, but it is presumed that part of the early formed nucleic acid is viral precursor. This is supported by the presence in infected *E. coli* of DNA containing hydroxymethylcytosine characteristic of the T_2 bacteriophage, by the presence of DNA in the cytoplasm of HeLa cells infected with vaccinia virus, together with the early sensitivity of that infectious sequence to inhibition by 5-fluorodeoxyuridine (a specific inhibitor of DNA synthesis; Salzman, 1960), and by the infectious nature of RNA extracted early in the infection with tobacco mosaic virus.

TABLE 1

VIRAL-INDUCED SYNTHESIS OF NUCLEIC ACIDS IN ANIMAL CELLS

Virus	Cell type	Maximum per cent increase per cell		Investigators
		RNA	DNA	
Poliovirus	HeLa	100	—	Ackermann *et al.* (1959) Barski (personal communication*)
Vaccinia	HeLa	30–105	—	Loh *et al.* (1959)
Pseudorabies	Rabbit kidney	—	35	Kaplan and Ben-Porat (1959)
Herpes virus	HeLa	—	100	Newton and Stoker (1958)
Adenovirus-4	HeLa	—	180	Ginsberg and Dixon (1959*a*)
Adenovirus-5	HeLa	Small	Severalfold	Boyer *et al.*

* Based on microspectrophotometric analysis of single cells.

However, in several systems where a net increase in nucleic acid could be observed (that is, with poliovirus, pseudorabies virus, adenovirus, vaccinia virus, and herpes virus), the quantities of the material formed after infection (TABLE 1) were found to be excessive in relation to the amount of virus ultimately produced. If the RNA formed in a single HeLa cell after infection with poliovirus were viral RNA, it would correspond to 10^7 virus particles (Ackermann, 1958), while the DNA produced in one cell infected with herpes virus was estimated to be equivalent to 10^6 virus particles (Newton and Stoker, 1958).

Further, in some instances a major part of the nucleic acid formed was not of the type found in the infecting agent, that is, RNA was produced in cells infected with a virus containing DNA (Ginsberg and Dixon, 1959*b*). The increases in DNA of cells infected with vaccinia virus revealed by cytochemical techniques (Alousi *et al.*, 1960) are difficult to follow by direct chemical analysis because of the small magnitude of the increase. In contrast, the same methods readily show increases in RNA of 30 to 100 per cent of the normal values for the host-cell cytoplasm. This synthesis occurring after infection has been confirmed also by a study of the rate of incorporation of radioactive-labeled

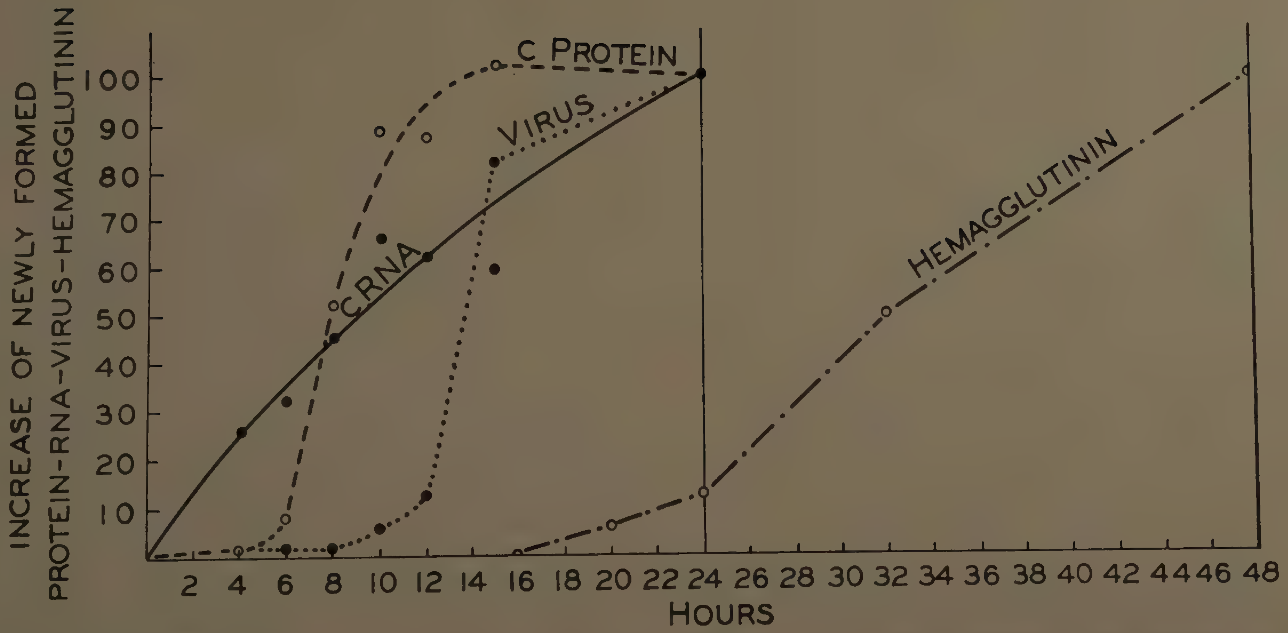

FIGURE 1. Replicate HeLa cell cultures were exposed to virus (93 PoFU/cell) for 4 hours. Excess virus was removed by washing with balanced salt solution. The cells were disrupted with Potter-Elvehjem homogenizer in citrate saline solution and separated into nuclea and cytoplastic fractions by differential centrifugation. The cytoplastic protein, RNA, and virus are plotted as the per cent of the 24-hour yield, hemagglutinin as the per cent of the 48-hour yield.

adenine into RNA (Joklik and Rodrick, 1959). A significant increase in the nuclear RNA of HeLa cells infected with type 5 adenovirus was also reported. However, the same cells show massive increases in DNA (Ginsberg and Dixon, 1959*b*). There is strongly suggestive evidence that this agent is also of the DNA type. By the use of tracer techniques, a considerable turnover of RNA was demonstrated in *E. coli* infected with T_2 or T_7 bacteriophage, but in these instances any net synthesis, if it occurs, is reported to be quite small relative to the DNA being synthesized (Volkin *et al.*, 1958; Volkin and Astrachan, 1956).

In these infections that ultimately result in cell death, the net increases of nucleic acid were reported to be confined to either the nucleus or cytoplasm, but were not found in both. Within the limits of this small series, synthesis of RNA has been reported in cells infected with viruses of the DNA type, but a net synthesis of DNA has not been reported for infection with agents of the RNA type.

Concept and Evidence for Functional Nonprecursal Viral RNA

Clearly, materials are formed after infection that do not constitute what is generally defined as the virus, that is, the agent as it exists in the extracellular state. Could this material constitute part of the agent as it exists in its functional or reproductive form? The DNA viruses at least might resemble higher organisms to the extent that they possess in part of their life cycle—in the intracellular state—a specific RNA as well as DNA. While proteins characteristic of infection but antigenically unrelated to host or virus have not been investigated systematically, they are not unknown. Presumably, as with higher organisms, the specific RNA and protein would arise from the functioning of the specific DNA. These nucleic acids with the various structural proteins and enzymes, 25 of which are known for one system described by Flaks, could constitute a relatively complex intracellular organism, albeit not one organized within a single membrane, other than that contributed by the host cell. In this concept the isolable agent bears a formal relation to an inactive spore.

The problem then of defining the intracellular virus is one of deciding what specificities of the structure present were imposed by the infecting agent and their essentiality to its reproductive process. One aspect is approached by isolation and physical characterization of the materials, the second by segregation of the various activities through the use of specific inhibitors. In the latter case interpretations are more easily made where differential sensitivity of the activities are found.

Inhibition of the synthesis of DNA has not been found to inhibit the replication of RNA-containing viruses (Fairman, 1959; Tamm, 1960*a* and *b*), but the converse has been amply demonstrated. For example, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole has been found to prevent the incorporation of adenine into RNA (Tamm, 1960*a* and *b*) and to inhibit the multiplication of vaccinia virus (Loh *et al.*, 1959), adenovirus, as well as viruses of influenza and poliomyelitis. For some years it has been known that treatment of infected cells with RNase would prevent the multiplication of viruses of both the DNA and RNA types (Burnet *et al.*, 1957; LeClerc, 1956; Tamm and Bablanian, 1960*b*).

While each of these experiments contains some limitations and are subject to other interpretations, together they strongly suggest that either RNA synthesis or the exact conditions necessary for it are essential to the replication of several DNA viruses.

Evidence for Viral-Induced Synthesis of Host-Cell Materials

In addition to such specific RNA that functions in virus reproduction but acts not as a material precursor, there is in several infections a considerable cellular content formed presumably as a result of either continued or stimulated functioning of the host cell. This may arise as a mere incidental accompaniment that must occur when conditions are rendered suitable for viral synthesis. The cell architecture may not allow the conditioning for nucleoprotein synthesis of an area small enough for production of virus exclusively. There is considerable evidence that the activity of the cell is not devoted exclusively to virus production after infection, even with agents ultimately producing necrosis.

The major part of the RNA produced after infection of cells with poliovirus is similar in its base ratios to host-cell RNA and dissimilar to viral RNA (Ackermann *et al.*, 1959; Ackermann, 1958). The increases in the glycolytic enzymes in the same system likewise clearly represent synthesis of cellular material during part of the infectious cycle of poliovirus (Matzelt *et al.*, 1958). Continued cellular activity at even a higher level of organization is suggested by the report of nuclear division occurring without cellular division in HeLa cells after infection with pseudorabies (Kaplan and Ben-Porat, 1959). Furthermore, cellular division of parasynchronized cultures of HeLa cells was observed after infection with either Newcastle disease virus or influenza virus (Wheelock and Tamm, 1959).

Also meaningful to the present thesis are experiments that show that the massive synthesis of DNA observed in the infection with pseudorabies is not essential for multiplication of that virus, although it is a usual accompaniment of the infection. Heavily irradiated cells provide a suitable environment for multiplication of pseudorabies, but presumably the damaged host-cell elements cannot function and the incidental DNA synthesis that is the major activity in the normal infection does not occur (Kaplan and Ben-Porat, 1959). Also significant is the report that in cell suspensions poliovirus could be grown without net increases in RNA and protein. Unfortunately the cell injury imposed was not defined as in Kaplan's experiments (Salzman *et al.*, 1959). The extent of synthesis of nonviral RNA in infections with either poliovirus or vaccinia virus is subject to certain variables that have not yet been suitably evaluated. The rate of development of the cytopathic effect may be an important factor and one responsive to the environmental conditions. Of course in any particular culture the accumulation of RNA is progressive only to a point, and cannot be demonstrated once cytopathic effects are so advanced that loss of cytoplasmic material has occurred (Rothstein and Manson, 1959; Maassab and Ackermann, 1959).

From a comparative viewpoint, the chemistry of the vaccinial infection that we will consider in some detail is of particular interest in that the agent contains DNA and develops at a cytoplasmic site in the cell. It should be possible to determine if the type of nucleic acid that undergoes hyperdevelopment depends upon the site of intracellular development, or is determined by the kind of nucleic acid found in the virus. The following data will show there is a considerable accumulation of RNA in the cytoplasm without a detectable net increase in the total cellular DNA. In this respect it superficially resembles the poliovirus that develops in the cytoplasm rather than the DNA-containing viruses that develop at a nuclear site.

The Nucleic Acid Composition of Ordinary HeLa Cells Under Growing and Resting Conditions

Since the generation time (20 hours) of HeLa cells is well within the period of infection studied, it is necessary to consider the changes in cellular constituents of the uninfected control cells under various conditions in comparison with changes induced by infection. Replicate cultures of HeLa cells kept under growing and resting conditions were analyzed over a 24-hour interval for their nucleic acid contents and number of cells. Resting conditions were produced by the use of maintenance medium (Scherer, 1953) and growth conditions with Eagle's medium (Eagle *et al.*, 1957).

HeLa cells adapted to growth in either Eagle's basal medium supplemented with 20 per cent human serum or 60 per cent Hank's balanced salt solution with 40 per cent human serum were used. On the third day after implantation the cultures containing Eagle's medium were changed to fresh growth medium containing 20 per cent equine serum instead of human serum, and those grown in balanced salt solution were changed to 90 per cent Scherer's maintenance solution with 10 per cent equine serum. These cultures were reincubated and used on the fourth day following implantation. The cultures kept in Eagle's medium and the others maintained in Scherer's medium were called growing and resting cells respectively.

When cells kept under resting conditions were analyzed at the end of a 24-hour incubation period at 37° C., no changes in total RNA, DNA, or cell count were observed (FIGURE 2). However, when the cells were kept under growing conditions for 24 hours at 37° C., increases in total RNA (100 per cent), DNA (20 per cent), and cell count (32 per cent) were observed. An alteration in the ratio of RNA-P to DNA-P was also observed for cells kept under growing conditions; however, such a change did not occur in cells kept under resting conditions.

The Effect of Vaccinia Virus on HeLa Cell Cultures Under Resting and Growing Conditions

When HeLa cells kept under growing or resting conditions were infected with vaccinia virus, certain alterations in cellular activity were observed that differed depending upon the conditions prevailing.

First, under growing conditions where the control cultures showed during a 24-hour interval increases in the number of cells (32 per cent), amount of nRNA-P (50 per cent) and nDNA-P (20 per cent), the infected cultures showed no significant changes (FIGURE 2) of this type after infection. However, the increases occurring in cRNA-P (50 per cent) and c-Protein (24 per cent) were upon infection further stimulated to 105 and 55 per cent respectively.

Thus, under growing conditions that allow for net increase in the large molecules of both cell fractions, infection with vaccinia inhibits the nuclear activities and stimulates the cytoplasmic ones.

Second, under resting conditions that ordinarily cannot support net increases of these materials in either fraction, infection induces a net synthesis specifi-

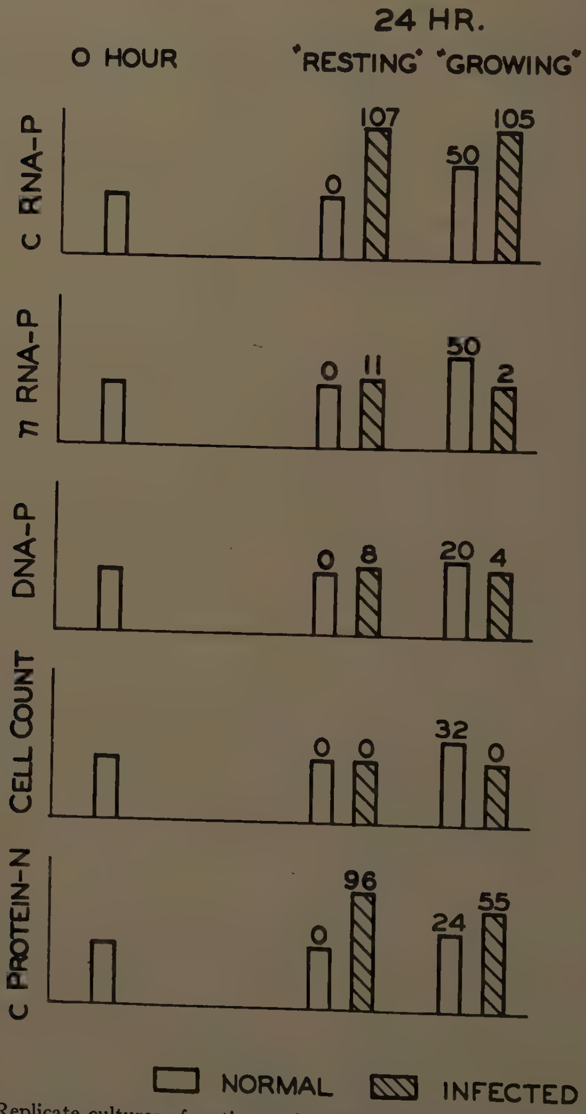

FIGURE 2. Replicate cultures of resting and growing cells were prepared as described in the text. From control and infected cultures taken at 0 and 24 hours, fractions containing the nuclei and cytoplasm were obtained by homogenization and centrifugation. The amounts of DNA, RNA, and protein per fraction of one cell found at zero time were assigned the value of 100. The per cent increases that occurred by 24 hours are indicated above the appropriate bars of the graph.

cally in the cytoplasm (c-RNAP 107 per cent and c-Protein 96 per cent). The magnitude of these activities is quite comparable to those that occur under growing conditions, but seem more impressive when compared to either a 24- or 0-hour control resting culture.

Kinetics of Net Synthesis of RNA, Protein, Virus, and Viral Hemagglutinin

The pattern of increase of cytoplasmic RNA, protein, virus, and viral hemagglutinin was determined in replicate cultures of cells at various times in the interval from the initiation of infection to the 24th hour. So that the characteristics of development of these materials that differ in absolute amount may be compared, the amount of each at the 24th hour was assigned the value of 100 and the amount at other times some proportion of 100. In this form, data concerning the newly formed protein and RNA of the cytoplasm, viral infectivity, and viral hemagglutinin are plotted in FIGURE 1. The increase of cytoplasmic RNA is detected as early as four hours after infection and can be represented by a shallow convex curve. It appears to accumulate at a decreasing rate during the latter part of the infectious period. However, increase in cytoplasmic protein is not detected until the fifth or sixth hour after infection, at which time it proceeds rapidly for the next several hours until, at about the 12th to 15th hour, it begins to level off.

Although the first increase in intracellular viral activity is not detected until 8 to 10 hours after infection, it proceeds rapidly for the next several hours and begins to reach maximum at about the 15th hour. When the patterns of increase for both cytoplasmic RNA and protein are compared to that obtained for the virus, it should be noted that they precede the first appearance of intracellular viral activity by several hours. It is also of interest to note that 50 per cent of the protein and RNA are formed before the appearance of any viral activity. The evidence of stained preparations shows a detectable accumulation of cytoplasmic DNA eight hours before the appearance of infectious activity.

Unlike the findings with poliovirus, the increases of RNA and protein in the cytoplasm of cells infected with vaccinia virus do not closely parallel each other with respect to time during the infectious sequence. Further, subfractionation of the cell cytoplasm and subsequent analysis of the cells infected with poliovirus revealed increases in the protein and RNA of each fraction, although not to equal extent. The same procedure, when applied to the vaccinal infection, showed that the increase in the RNA content of the cytoplasm is accounted for largely by changes in the cell sap, while the increases in protein occur in the fraction containing the small particles.

The data in TABLE 2 were obtained from an analysis for RNA of fractions prepared from cells before infection and 17 and 41 hours after infection. Control cultures uninfected but of the same age were also treated in the same manner.

Cells were disrupted in citrate-saline containing versene with the Potter-Elvehjem homogenizer. The nuclear fraction was removed by centrifugation at 2000 g for 10 min., while the small particles were sedimented by a force of 100,000 g applied for one hour. Presumably the cell sap so prepared contains the very low molecular weight RNA associated with amino acid activation, while the small particles contain the RNA of the microsomes.

It will be noted there was an increase of nearly 40 per cent in the total RNA per cell in both infected and uninfected cultures. This increase in RNA that occurred by 41 hours in the control culture was largely sedimentable with the nuclear fraction, while that of the infected cell was found largely in the cell sap fraction where the increase was 90 per cent greater than the value before infection.

In the same experiment, when protein determinations were made, the increases in protein occurred only in the infected culture and are accounted for largely by a 100 per cent increase in the small particles (TABLE 3).

TABLE 2

RIBONUCLEIC ACID CONTENT* OF FRACTIONS OF NORMAL AND VACCINIA-INFECTED CELLS

Infected				Hours after infection	Uninfected			
Cell sap	Small particles	Nuclei	Total		Cell sap	Small particles	Nuclei	Total
10.49	17.15	11.17	38.81	0	10.49	17.15	11.17	38.81
17.06	22.31	9.06	48.43	17	13.44	20.25	14.32	48.01
20.05	22.44	10.27	52.76	41	13.22	20.00	27.31	60.53

* Expressed as μgm. of RNA phosphorus × 10^{-10} per fraction obtained from one cell.

TABLE 3

PROTEIN CONTENT* OF FRACTIONS OF NORMAL AND VACCINIA-INFECTED CELLS

Infected				Hours after infection	Uninfected			
Cell sap	Small particles	Nuclei	Total		Cell sap	Small particles	Nuclei	Total
111.3	46.08	79.38	236.8	0	111.3	46.08	79.38	236.8
143.4	75.60	65.60	284.6	17	122.8	58.00	63.84	244.6
127.9	90.78	71.42	290.1	40	96.38	53.30	94.98	244.7

* Expressed as μgm. × 10^{-9} of protein per fraction obtained from a single cell.

It is conceivable that the increases in the small molecular weight RNA induced by infection have a function in the synthesis of the protein found subsequently in the small particles.

The data of TABLES 1 and 3 were taken from a single experiment. It is of particular interest to note that, while the trend of such experiments with vaccinia virus is reproducible, the quantitative aspects are not. The inhibition of increase of RNA and protein in the nucleus is consistent, but the magnitude of the incremental protein and RNA of the cytoplasm is quite variable from one experiment to another. It may be concluded from the data, as with those cited previously for other virus systems, that this hyperdevelopment of part of the cell is more incidental to than essential for virus production. As with the carcinogenic viruses, there is some suggestion that an inverse relation may exist. Environmental conditions in tissue culture that seem to favor production of polyoma virus also favor a marked necrotic type of cytopathic effect.

The environmental factors important to the phenomenon have not as yet been elucidated clearly. The findings suggest that the virus interferes with an important controlling mechanism in the cell that has not been encountered yet in other connections.

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INTERFERON AS A CHEMICAL INTERMEDIARY IN VIRAL INTERFERENCE*

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Until recently, viral interference was relegated to the limbo of obscure phenomena of little biological significance. Its experimental resurrection can be attributed to two principal factors: the advent of considerably improved techniques for quantitative assay of viruses and the discovery and partial characterization of interferon by Isaacs and his colleagues.[1,2] The availability of these methods has stimulated many investigators, each using his own favorite virus-cell system, to re-evaluate the role of interference in acute and chronic viral infections. It is our contention that these studies will provide considerable insight into the nature of viral infections and of certain biochemical factors concerned with cellular resistance.

In a recent review article[3] an attempt was made to analyze some of the data and theories that have been put forth to explain the biological basis of viral interference. The present report deals more extensively with our current theoretical concepts of the interference phenomenon. It seems profitable—even at this early stage of research activities in this field—to attempt a statement of the problems, to cite some of the experimental evidence for our present viewpoints, and to indicate future directions for potentially fruitful research. In order to state the problems more clearly we have taken the liberty of using a series of oversimplified diagrams to represent the stages of cellular infection and viral interference. For purposes of orientation the three readily recognizable events that occur during infection of a single cell with a single virulent virus particle can be expressed in the following way:

$$\text{Cell} + \text{V} \rightarrow (\text{Cell}\cdot\text{VNA}) \rightarrow \text{Cell}^* + \max \text{V}$$

The first reaction in the infectious process represents attachment and penetration of virus; the second symbolizes virus eclipse or assimilation of virus nucleic acid (VNA) into the genetic apparatus of the cell; and the third illustrates the two measurable products of the reaction, a dead cell (cell*) and a maximal yield of virus progeny (max V).

When two distinguishable viruses infect the same cell that is susceptible to infection with either alone, it has been presumed that the first two events in the infectious process will be similar. If one of the viruses is capable of interfering with the other, the reaction may be written as follows:

$$\text{Cell} + \text{V}^{i} + \text{V}^{s} \rightarrow (\text{Cell}\cdot\text{VNA}^{i}\cdot\text{VNA}^{s}) \rightarrow \text{Cell}^{r} + \min \text{V}^{s}$$

This representation of viral interference implies that both the interfering (V^{i}) and superinfecting (V^{s}) viruses adsorb on and penetrate the cell.[4,5] Evidence

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for the formation of the intermediate complex (Cell·VNA^i·VNA^s) is less convincing, but Isaacs and Edney[6] have demonstrated that superinfecting influenza virus "disappears" after adsorption on "interfered" cells. For purposes of this discussion, let us assume that interfering virus has been rendered noninfectious by ultraviolet irradiation and cannot stimulate the cell to produce V^i progeny. Under these circumstances, the cell survives and the measurable products of the reaction are a resistant cell (cell^r) and a reduced yield of virus progeny (min V^s).

Most of the former theories of viral interference have been concerned with the nature of the intermediate complex (Cell·VNA^i·VNA^s) depicted in the above diagram. It has been suggested that interference occurs if the nucleic acid moieties of two viruses within the same cell compete with each other. This competition has been considered to be either genetic, in which the nucleic acid of the interfering virus is thought to pre-empt "chromosomal" sites for attachment of the superinfecting virus, or metabolic, in which the two virus nucleic acids compete for an essential enzyme or other cellular constituent.[7]

Neither the genetic nor the metabolic theory has been substantiated, and considerable doubt has now been cast on the validity of the concept that interference represents simple competition between two incompatible virus nucleic acids. In fact, the discovery of interferon suggests that viral interference is not necessarily a simple two-virus one-cell system. There is now ample proof that, under certain circumstances, one virus can stimulate a cell to release a soluble nonviral substance that, when transferred to a second cell, can render it resistant to infection. Based on this evidence, it would now appear that interference takes place in a complex series of events mediated by a nonviral cellular product, interferon. This finding has prompted us to propose an alternative theory in which interference can be depicted as occurring in three stages:

$$\text{Stage 1: Cell}_1 + \text{V}^i \rightarrow (\text{Cell}\cdot\text{VNA}^i) \rightarrow \text{Cell}^r + \text{I}$$

$$\text{Stage 2: Cell}_2 + \text{I} \rightarrow (\text{Cell}\cdot\text{I}) \rightarrow \text{Cell}^r$$

$$\text{Stage 3: Cell}^r + \text{V}^s \rightarrow (\text{Cell}\cdot\text{VNA}^s) \rightarrow \text{Cell}^r + \text{min V}^s$$

In many interference systems these three stages are not clearly separable but the reactions proceed *pari passu*. However, in order to subject the theory to critical analysis it is necessary to examine each reaction separately. The first stage illustrates the production of interferon (I) by a cell exposed to interfering virus (V^i). If the infected cell releases interferon into the extracellular fluid or tissue culture medium, the interferon is then free to react with another susceptible cell. This is the event depicted in Stage 2 in which interferon adsorbs on another cell and forms a stable cell-interferon (cell·I) complex. In this process interferon is used up and the product of the reaction is a resistant cell (designated cell^r). It is well to keep in mind that interferon itself is not capable of stimulating the cell to produce more interferon.[1] When the superinfecting virus (V^s) is introduced into the system (Stage 3), it adsorbs on the resistant cell and presumably is eclipsed. The product of this reaction is a reduced yield of superinfecting virus (min V^s).

Biological and Physicochemical Properties of Interferon

With this outline in mind the crucial first question is whether there is proof that interferon exists as a distinct biological and chemical entity. It is necessary to state from the outset that the evidence is purely circumstantial, but increasing numbers of laboratories are reporting that "soluble" products of infected cells possess interfering properties similar to those of interferon. To cite but a few examples, interferonlike substances have been found capable of transmitting resistance to infection with the following viruses: influenza,[2] vaccinia,[8] poliovirus,[9] vesicular stomatitis,[10] eastern equine encephalomyelitis (EEE),[3] western equine encephalomyelitis,[11] and a variety of other arbor viruses.[12] In our studies interferon is prepared in chick embryos injected with infectious influenza virus. The allantoic fluid is harvested 72 hours after infection and then subjected to several one-hour cycles of centrifugation at 100,000 g. The supernatant fluid, which contains approximately 100 EID_{50} of residual virus, is our source of interferon. The bioassay method used for titration of interferon is based on inhibition of plaque formation in monolayer cultures of chick embryo fibroblasts infected with EEE virus. The end point of the titration is taken as the twofold dilution of interferon that reduces the number and size of plaques by approximately one half of the calculated input. This has proven to be a relatively sensitive assay method with a titration error of about 50 per cent.

The method by which our interferon is prepared constitutes the primary evidence of its existence as an entity distinct from mature influenza virus. Infected allantoic fluid from which 99.9999 per cent of the virus has been removed by centrifugation has exactly the same interfering activity as the original fluid. There are four additional lines of evidence that interferon is not a known protein constituent of influenza virus: (1) its activity is not neutralized by anti-V antibody; (2) its activity is not neutralized by anti-S antibody; (3) it does not adsorb on chicken erythrocytes as does V antigen; and (4) it is more stable to heat than is the interfering influenza virus itself.

Certain of the physicochemical properties of interferon also suggest that it is a unique product of the virus-infected cell: (1) interferon is nondialyzable; (2) it is moderately heat stable (only 90 per cent of its biological activity is destroyed at 85° C. for one hour); (3) it is extraordinarily stable over a *p*H range from 1 to 12.5; (4) it is precipitated only by relatively high concentrations of ammonium sulfate and methanol, and (5) its activity is destroyed by trypsin and chymotrypsin but not by RNase or DNase.

From these data we infer that interferon is a protein (or at least contains a protein) and, because of its extraordinary resistance to hydrolysis by acid or alkali, that it is a basic protein. The main difficulty that we have had in further characterizing interferon has been our inability to purify it. It adsorbs readily on *N*,*N*-diethyl-amino-ethyl (DEAE) and carboxymethyl cellulose resins but cannot be eluted by any of the standard methods. Interferon also adsorbs on bentonite and approximately 25 per cent of the biological activity can be eluted at *p*H 9 or above. This latter finding is further evidence, perhaps, that interferon is a basic protein.*

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* D. C. Burke (personal communication) has informed us of his success in purifying interferon by column chromatography. The isoelectric point of his purified preparation is esti-

Preliminary studies by ultracentrifugal analysis suggest that this technique may provide valuable information. As previously mentioned, none of the biological activity of interferon is sedimented by prolonged centrifugation at 100,000 g. To achieve a significant reduction in titer it is necessary to subject a suspension of interferon to centrifugation at 60,000 rpm (254,000 g, average) for two hours. These studies indicate that the sedimentation constant for interferon is probably in the range of 2 Svedberg units. More definitive data on sedimentation constants will depend on the availability of a more accurate bioassay method. Nevertheless, it would appear that interferon is a relatively small molecule.

Production of Interferon

In our theory of the mechanism of interference it is presumed that interferon is a nonviral product of the virus-infected cells. Isaacs' original studies were carried out with irradiated noninfectious influenza virus as the interfering agent. However, it has been shown subsequently that infectious virus is also capable of stimulating a cell to produce interferon. Let us rewrite our expression for the Stage 1 reaction accordingly:

$$\text{Cell} + \text{V}^{\text{i}} \rightarrow (\text{Cell}\cdot\text{VNA}^{\text{i}}) \rightarrow \text{Cell}^{\text{r}} + \text{V}^{\text{i}} + \text{I}$$

The evidence that the same cell can produce virus and interferon comes from studies of influenza virus infection of chick embryos. In these experiments tremendous quantities of infectious influenza virus were formed by allantoic cells before any interferon could be detected in the allantoic fluid. It seems logical to assume that virtually every allantoic cell was infected. However, it is less certain that infectious influenza virus, rather than thermally-degraded virus, served as the stimulus for interferon production. Further studies are required to clarify this point. The above diagram also implies that the intermediate complex ($\text{Cell}\cdot\text{VNA}^{\text{i}}$) is essential for the formation of interferon. No direct experimental evidence is available to support this contention, but it is known that incomplete influenza virus deficient in nucleic acid[13] has only limited interfering activity.[14] Proof that virus nucleic acid can induce the cell to produce interferon may possibly be obtained by studying the effect of phenol-extracted virus RNA. Further data are also required before interferon production can be equated with establishment of interference (cell^{r}). Such evidence could be obtained by studying the kinetics of ultraviolet inactivation of the interfering and interferon-producing properties of influenza virus. If the same dose of irradiation destroyed both properties, the assumption could be made that they are identical and that both reside in the RNA components of the virus.

Cell-Interferon Interaction

The presumed mechanism by which interferon, in the absence of interfering virus, renders a cell resistant to superinfection is shown by the reaction for Stage 2 of our theory:

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mated to be in the range of *p*H 4.5 to 5.0, a finding which negates our hypothesis that interferon is a basic protein. It is of interest that his data on ultracentrifugal analysis reveal a sedimentation constant that is only slightly higher than our estimate based on bioassay.

$$\text{Cell} + \text{I} \rightarrow (\text{Cell}\cdot\text{I}) \rightarrow \text{Cell}^{r}$$

This diagram postulates that interferon attaches on cells, forms a stable cell-interferon complex, is used up in the reaction, and that the cell becomes resistant to infection. As indicated by Lindenmann *et al.*,[2] interferon does not readily adsorb on the surface of allantoic membranes in suspended tissue cultures. We have been able to study the adsorption reaction only by placing a small volume of concentrated interferon in contact with a monolayer of approximately 2×10^7 chick fibroblasts. When this is done, the cells gradually take up interferon over a one-hour period but not at an exponential rate. At the end of the adsorption period, the cells were washed and disrupted by alternate freezing and thawing. No interferon could be recovered from the disrupted cells, indicating the formation of a firm union between cells and interferon.

Additional evidence for interaction between cell and interferon was sought by comparing the rate of adsorption and the rate of conversion of cells to a resistant state (cell^{r}). Although the correlation was far from perfect, the general thesis can be advanced that the degree of resistance to infection with EEE virus depends on the amount of interferon adsorbed. This resistance persists as long as cells remain in contact with interferon. If, however, the cells are washed free of residual interferon and incubated in an interferon-free medium, they gradually recover their susceptibility to infection. It seems likely that the cell-interferon complex is quite stable and that reversion to susceptibility is associated with formation of new cells.[15] However, further studies with stable cell lines are required to determine the duration of interferon action.

The Resistant Cell

Let us next examine the presumptive mechanism by which the interferon-treated cell resists infection, as exemplified by Stage 3 of our theory:

$$\text{Cell}^{r} + \text{V}^{s} \rightarrow (\text{Cell}\cdot\text{VNA}^{s}) \rightarrow \text{Cell}^{r} + \text{min V}^{s}$$

Once again, no definitive proof is at hand for assuming that the nucleic acid of the superinfecting virus enters the resistant cell and is eclipsed (Cell·VNA^{s}). Nevertheless some evidence is available to indicate that this event is likely. We have studied the rates at which superinfecting EEE virus adsorbs on susceptible cells and on cells rendered resistant by interferon.[3] No difference could be detected, which suggests that interferon does not alter the surface receptors for attachment of EEE virus to chick fibroblasts. Additional indirect evidence that interferon acts inside the cell was obtained by studying one-step growth curves of EEE virus.[3] It was found that interferon effectively reduced the virus yield even if the cells were treated one hour after infection, at a time when most of the infecting EEE virus could be presumed to be intracellular or at least cell-associated. This suppressive effect of interferon on virus production is not merely due to inhibition of virus release. Disrupted cells contain no more infectious virus than does the culture medium.

These experimental results suggest that interferon acts by preventing the maturation of superinfecting virus within the resistant cell. If this is the case, the availability of this interfering agent, even in an impure state, should open up potentially fruitful fields for further investigation. It would be extremely interesting, for example, to determine whether interferon arrests virus replication at a particular stage of its intracellular development or whether one of the components of the virus fails to form within the resistant cell. Among the possible explanations of mechanisms of interferon action, the following appear to deserve special consideration:

(1) The resistant cell may be unable to synthesize virus nucleic acid. In this situation no template would be available for production of virus protein.

(2) The resistant cell may remain competent to synthesize virus nucleic acid, but formation of virus protein may be inhibited.

(3) Both virus nucleic acid and protein may be formed, but the resistant cell may be incapable of assembling the two constituents to make mature virus.

The last of these three possibilities is most amenable to experimental confirmation. Studies with fluorescein-labeled antibody or with other immunologic methods should reveal whether the resistant cell is still capable of making virus protein. A more imposing problem is the question of whether the resistant cell can synthesize virus nucleic acid. It is possible that significant data could be obtained by direct chemical analysis, by rates of P^{32} incorporation into nucleic acid, or by microspectrophotometry. However, these techniques do not readily permit a distinction between the nucleic acids of viruses and cells. Phenol extraction methods[16] may possibly provide a means for identification of the infectious RNA of EEE virus within resistant cells, but the obstacles to a quantitative analysis are formidable.

Histology of the Resistant Cell

In addition to suppressing virus production, interferon protects the cell against the cytopathic action of EEE virus. It seems logical to assume that these effects are related. Preparatory to embarking on quantitative and definitive studies of nucleic acid synthesis, it seemed worthwhile to determine whether any information could be obtained by indirect histochemical methods. To this end we chose the acridine orange technique for staining nucleic acids of uninfected and infected cells.[17] When cells are stained with acridine orange and examined under a microscope equipped with an ultraviolet light source, nuclear DNA fluoresces a bright yellow and cellular or nucleolar RNA stains orange-tan or red. Mitotic figures or other cell structures can be readily identified in uninfected chick embryo fibroblasts grown on cover slips in Leighton tubes. Cells grown under the same conditions in the presence of large quantities of interferon revealed a normal distribution of DNA and RNA. The presence of abundant mitotic figures in interferon-treated cells suggested that nuclear division was not inhibited.

The sequence of events observed in chick fibroblasts infected with EEE virus and stained with acridine orange was not unlike that described by Armstrong and Niven[17] for other viral infections. Five hours after infection with EEE virus the cells showed a tendency toward cytoplasmic vacuolization and clumping of nuclear chromatin. These effects progressed to cytoplasmic degeneration and margination of nuclear chromatin. At 20 hours after infection marked cytolysis was apparent, the nuclei had lost much of their capacity to stain for DNA, and red granules of RNA were extruded from the cell (FIGURE 1). By

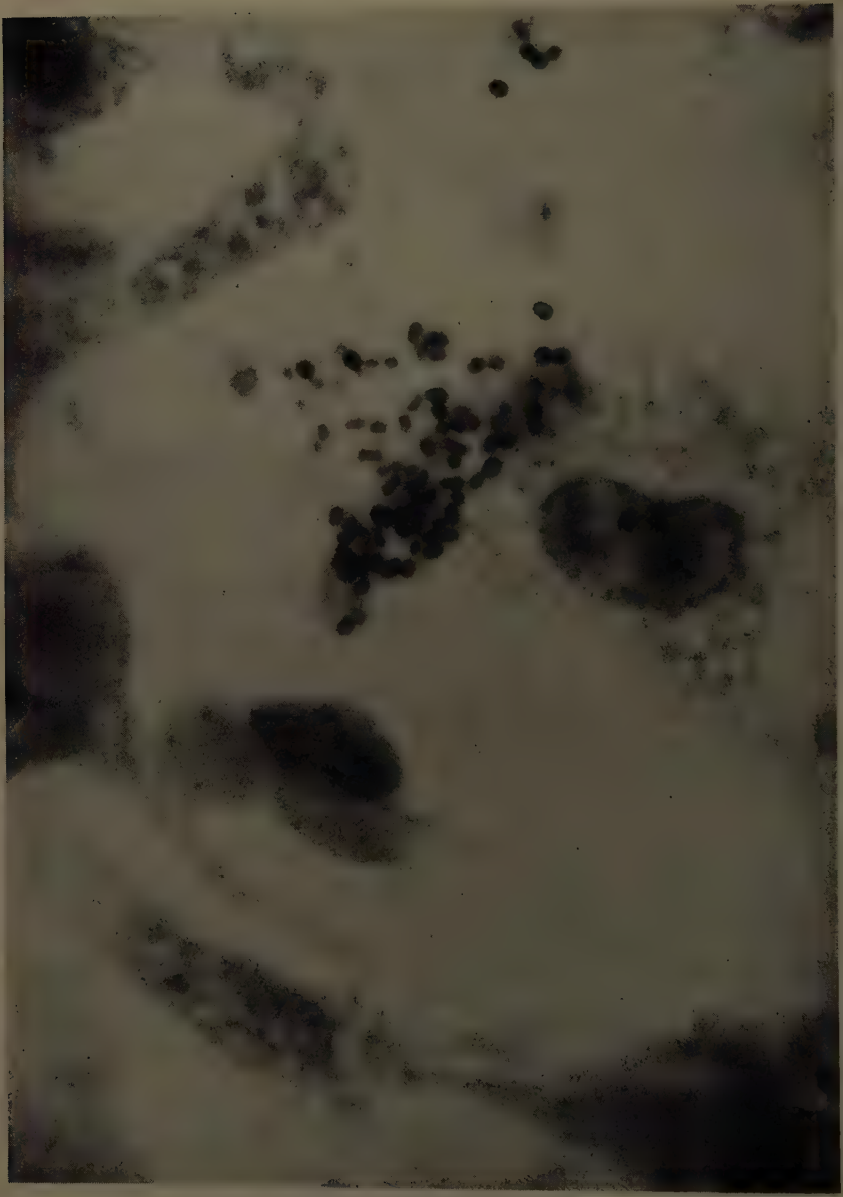

FIGURE 1. Chick fibroblasts infected 20 hours previously with EEE virus and stained with acridine orange. The cytoplasm is undergoing degeneration and orange-red granules of RNA are lying outside the cell. The altered staining pattern of nuclear DNA is characterized by clumping and margination of chromatin. Negative print, ×512.

48 hours all of the cells in the culture had detached from the glass and were floating in the medium.

Interferon had a striking effect on the histological appearance of chick fibroblasts infected with EEE virus. The cell architecture remained well preserved, the distribution of RNA and DNA was indistinguishable from that of normal cells, and only rarely could extracellular RNA granules be detected. Despite the viral infection, mitotic figures could readily be seen in cells pretreated with interferon. In fact, at 48 hours the infected cells formed a tightly packed sheet in which numerous cells were noted to contain as many as twelve nuclei (FIGURE 2). These multinucleated cells were readily identifiable as fibroblasts. It has not yet been determined whether enhanced formation of multinucleated cells is caused by the action of interferon alone or in combination with superinfecting EEE virus. Also deserving of passing mention is the fact that nuclei of multinucleated cells are significantly smaller than nuclei of normal mononucleated fibroblasts.

Autointerference

Most of the foregoing discussion has centered about the production of interferon by one cell and the transfer of resistance by exogenous interferon. It is evident that the original cell that produces interferon also acquires resistance to superinfection. As shown by the diagram representing Stage 1 of our revised theory, the products of interaction between interfering virus and a cell may be a reduced yield of interfering virus, interferon, and a resistant cell. It seems possible that production of endogenous interferon is responsible for the resistance. Not only would this cell resist superinfection, but it also might limit multiplication of the original interfering virus. Stated in another way, the same virus particle that stimulates the cell to produce interferon may itself be inhibited within the resistant cell. Such an hypothesis would reduce all the events occurring during interference to a one-virus one-cell system. Whether a cell will respond to infection by production of virus progeny, interferon, or both products should be a function of cellular susceptibility to a particular virus. Three alternative pathways of infection are suggested:

$$\text{Cell} + \text{V} \rightarrow (\text{Cell}\cdot\text{VNA}) \rightarrow \begin{array}{l} (1)\ \text{Cell}^{*} + \max \text{V} \\ (2)\ \text{Cell}^{r} + \text{I} \\ (3)\ \text{Cell} + \min \text{V} + \text{I} \end{array}$$

In Reaction 1 rapid multiplication of a virulent virus in a highly susceptible cell will result in cell death before interferon can be produced. In Reaction 2 virus infectivity is impaired or the cell is intrinsically insusceptible and will produce interferon rather than virus progeny. If the reaction goes in the direction of pathway 3, the cell is presumably of intermediate susceptibility and may produce both virus progeny and interferon. Ostensibly, the outcome of this infection will depend on the intracellular equilibrium between virus and interferon. If virus is produced more rapidly than interferon, the cell will eventually die. If, however, accumulation of endogenous interferon exceeds virus production, the infection may not progress to cell death.

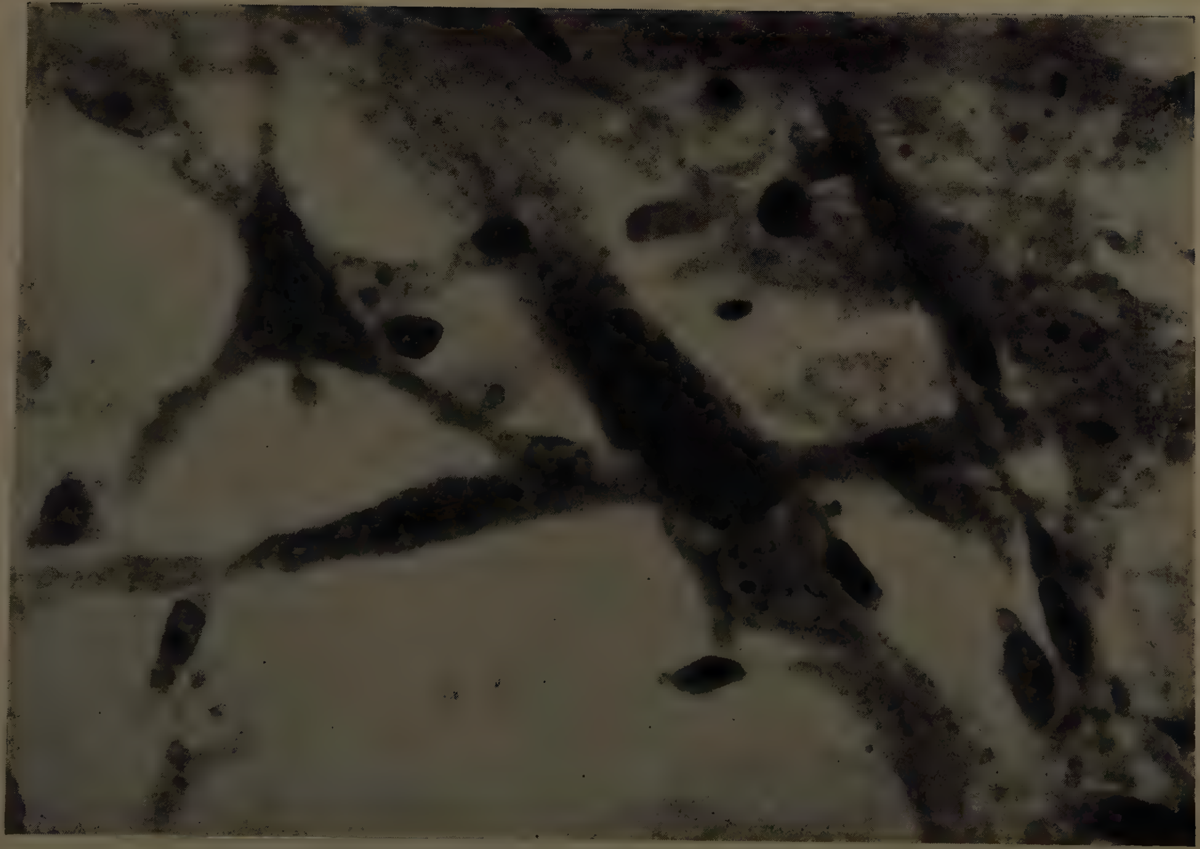

FIGURE 2. Chick fibroblasts exposed simultaneously to interferon and EEE virus 48 hours prior to staining with acridine orange. The distribution of RNA and DNA is not significantly different from uninfected cultures. Note the multinucleated fibroblasts. In the absence of interferon, infected cultures showed complete destruction of cells at 48 hours. Negative print, ×201.

Summary

A hypothetical mechanistic concept of viral interference has been presented as a working model for further experimentation. This theory is based on the postulate that interference occurs in three stages: (1) production of interferon by a cell infected with interfering virus; (2) cell-interferon interaction resulting in a resistant cell; and (3) suppression of multiplication of superinfecting virus within the resistant cell. Presumably, these events can occur in a single cell infected with a single virus particle, or interferon released by one cell can transmit resistance to a second cell. A more complete (and more accurate) biological interpretation of this phenomenon will depend on chemical characterization of interferon, knowledge of its intracellular site of action, and determination of its effect on the stages of virus replication.

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Discussion of the Paper

Emilio Weiss (*Naval Medical Research Institute, Bethesda, Md.*): In the last table you have given an example of a cell producing interferon and limiting its own virus production. Is there any evidence that this is the mechanism responsible for the host-virus balance that prevails in chronically infected cell cultures?

Wagner: This is an extremely interesting question that is under active investigation in several laboratories. Henle and his colleagues[1] have found that chronic myxovirus infection of MCN cells appears to be associated with an interferon that is capable of rendering these cells resistant to superinfection with vesicular stomatitis virus. Persistence of western equine encephalomyelitis virus in L cells is not inconsistent with production of interferon in these chronically infected cultures.[2] In trying to establish this point, the difficulty encountered is the insensitivity of the bioassay methods for detecting the presence of interferon. This is not surprising when it is recalled that, even under ideal conditions, large numbers of cells exposed to a high multiplicity of virus produce only small amounts of interferon. In chronically infected cultures we are dealing ordinarily with a small number of cells infected with very few virus particles at any one time. In addition, any interferon produced by a small proportion of the cells can probably attach irreversibly to the remaining uninfected cells in the culture, thus rendering them insusceptible to infection. Therefore, the only statement that can be made at present is that no inconsistency can be found in the thesis that interferon may be responsible for establishment of a viral carrier state in tissue culture. As suggested in a previous publication,[3] it seems possible that cyclic production of virus and interferon may be responsible for the host-virus balance that prevails in some chronically infected cell cultures.

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